The association of early childhood nutrition and growth with the intestinal microbiome and pneumonia

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Declaration

I, Shrish Budree, hereby declare that this thesis is my own work, both in concept and execution, apart from the normal guidance received from my supervisors and contributions by those acknowledged. This thesis is presented in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in the Department of Paediatrics and Child Health, Faculty of Health Sciences, University of Cape Town.

The thesis includes four manuscripts (two published and two under peer review at the time of submission) that are included as literal and word-for-word renditions of the published works and submitted manuscripts under peer review, as per general provision 6.7 in the General Rules for the Degree of Doctor of Philosophy (PhD) of the University of Cape Town. The text and data are presented as per the published articles, but tables and figures have been re-numbered for clarity and internal coherence. I was the first and corresponding author of all four publications. I collected, cleaned, and analysed all data, and drafted the manuscripts.

I confirm that I have been granted permission by the University of Cape Town's Doctoral Degrees Board to include the following publications in my PhD thesis, and where co-authorships are involved, my co-authors have agreed that I may include these publications.

Chapter 2 describes a longitudinal evaluation of growth and the prevalence of malnutrition and factors associated with poor growth in infants residing in a peri-urban, low income setting in South Africa. The published paper is:

- Budree, S., Stein, D. J., Brittain, K., Goddard, E., Koen, N., Barnett, W., Myer, L., & Zar, H. J. (2017). Maternal and infant factors had a significant impact on birthweight and longitudinal growth in a South African birth cohort. *Acta Paediatrica, 106*(11): 1793–1801. Doi: 10.1111/apa.14015

Contribution of student and co-authors: During the drafting of this manuscript, I was the medical officer on the Drakenstein Child Health Study (DCHS). I modified the anthropometry Case Report forms for the study to optimise the quality of data collected and implemented anthropometry training of study nurses and field workers. I cleaned and analysed the growth and nutrition data with the assistance of KB and LM. I performed all microbiome statistical analysis. I interpreted the data and drafted the manuscript under the guidance of my supervisor, HJZ. I, together with HJZ, conceived and designed the study. NK and DJS contributed psychosocial data and contributed to review of the manuscript. EG and WB contributed to the study design and reviewed the manuscript.

Chapter 3 investigates infant feeding practices and patterns, including weaning and introduction of complementary feeds, which are important determinants of growth, nutritional status, the microbiome composition, and risk of infection. The published paper is:

- Budree, S., Goddard, E., Brittain, K., Cader, S., Myer, L., & Zar, H. J. (2017). Infant feeding practices in a South African birth cohort—A longitudinal study. *Maternal & Child Nutrition, 13*(3), e12371. Doi: 10.1111/mcn.12371

Contribution of student and co-authors: Together with HJZ, I conceptualised this study. I cleaned and analysed the data with KB and LM. I drafted the manuscript. EG and SC contributed to the study design and reviewed the manuscript.

Chapter 4 presents a longitudinal analysis of the gut microbiome in children residing in a low income setting with stunting. The submitted paper is:

- Budree, S., Claassen-Weitz, S., Osman, M., Vatanen, T., Nicol, M. P., & Zar H. J. Evaluating the gut microbiome in children with stunting: Findings from a South African birth cohort. Submitted to *Med* (Cell Press Journal).

Contribution of student and co-authors: Together with HJZ and MPN, I conceptualised this study. As the medical officer on the DCHS, I was instrumental in designing the standard operating procedures for stool collection, transportation, and storage in the study. SC performed the wet-lab prep and initial 16S rRNA gene amplicon sequencing of stool samples. I processed the raw sequence data and performed the downstream bioinformatic analysis under supervision of TV. MO contributed to study design and manuscript review.

Chapter 5 provides a novel analysis of the gut microbiome in children residing in a low- and middle-income country (LMIC) with pneumonia compared with non-pneumonia controls. The submitted paper is:

- Budree, S., Claassen-Weitz, S., Osman, M., Vatanen, T., Nicol, M. P., & Zar H. J. Gut microbiome dysbiosis precedes the onset of childhood pneumonia: Findings from a South African birth cohort. Submitted to *Pediatrics* (American Association of Pediatrics Journal).

Contribution of student and co-authors: Together with HJZ and MPN, I conceptualised this study. SC performed the wet-lab prep and initial 16S rRNA gene amplicon sequencing of stool samples. I processed the raw sequence data and performed the downstream bioinformatic analysis under supervision of TV. MO contributed to study design and manuscript review.

Together the chapters form a thematically coherent and substantive body of work that addresses the specific aims and objectives of this thesis. The research included subsets of children enrolled in a South African birth cohort. This work advances the understanding of the relationship between nutritional status, the microbiome, and childhood pneumonia, and may help identify novel preventative and therapeutic strategies to improve health outcomes in early life in LMICs.

No part of the thesis has been submitted, is currently being submitted, or is to be submitted for a degree at this or any other university. I grant the University of Cape Town free licence to reproduce the thesis in whole or part for the purpose of research.

Signature: Date: 13 April 2025

Shrish Budree

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Abstract

Background

Malnutrition and pneumonia contribute significantly to under-5 mortality. The gut microbiome is associated with development of malnutrition and pneumonia. However, data on longitudinal growth, infant feeding practices, and the microbiome in children with malnutrition and pneumonia in low- and middle-income countries (LMIC) are scarce. This work aimed to investigate growth in early childhood, and associations of the microbiome with growth and pneumonia in an LMIC.

Methods

A prospective study of growth, pneumonia, and the microbiome was performed in the Drakenstein Child Health Study (DCHS), a birth cohort in a low-income setting in South Africa. Mothers enrolled antenatally, and mother-child pairs were followed through birth and early childhood. Comprehensive health, socio-demographic, nutritional, and psychosocial data were collected longitudinally from birth through 3 years. Anthropometry was measured by trained study staff. Dietary information was collected using food-frequency questionnaires. Active surveillance for World Health Organization (WHO)-defined pneumonia was undertaken. Longitudinal stool samples were collected at study visits and during pneumonia episodes. WHO z-scores were calculated, and malnutrition classified as stunted, wasted, or overweight/obese. Dietary data were analysed using WHO infant and young child feeding indicators. Microbiome 16S ribosomal RNA (rRNA) gene amplicon sequencing was performed and bioinformatic analysis conducted in QIIME2 and Phyloseq (R packages).

Results

Subsets of children in the DCHS were analysed. Growth analysis among 792 infants (50% female; 15% preterm) showed that birth weight was a significant determinant of growth ($p<0.001$). Stunting was highly prevalent (17% and 13% at 2 and 12 months, respectively). Feeding practices evaluated in 1076 infants (50% female, 17% preterm) showed low exclusive breastfeeding rates (13%), and high consumption of inappropriate foods (high sugar or high-fat food) at 1 year (91%). Microbiome analysis of 138 children aged 1–40 months (49% females) included 90 children with pneumonia (10% hospitalised) and 48 non-pneumonia controls. Stunted children had low diversity ($p=0.009$) and significant depletion in *Ruminococcus* ($q=0.03$). Compared with age-matched controls, dysbiosis was found during pneumonia episodes and before the onset of pneumonia, characterised by significant enrichment in (Escherichia coli) *E. coli* ($q=0.04$).

Conclusion

Malnutrition and poor infant feeding practices were highly prevalent despite strong primary healthcare programs. Correction of microbiome disruption may improve outcomes in pneumonia and malnutrition.

Keywords

Malnutrition

Pneumonia

Gut Microbiome

Infant Feeding Practices

Stunting

Dysbiosis

Low- and Middle-Income Countries (LMIC)

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While my educational journey has temporarily taken me away, my heart lies in Africa and in improving the health of children in Africa. I will continue to strive to better maternal and child health through cutting edge research, innovation and the development of novel therapeutics to push the boundaries of disease prevention and cure.

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Abbreviations

AGA: appropriate for gestational age

BMI: body mass index

BMIZ: body mass index for age z score

DBM: double burden of malnutrition

DCHS: Drakenstein Child Health Study

EBF: exclusive breastfeeding

FFQ: food frequency questionnaires

GA: gestational age

HFAZ: height-for-age z score

HIV: Human immunodeficiency virus

HMO: human milk oligosaccharides

IYCF: Infant and Young Child Feeding indicators

IPV: intimate partner violence

IQR: interquartile range

IRF: consumption of iron rich foods

LBW: low birth weight

LMIC: low and middle-income countries

LPS: lipopolysaccharide

LTFU: loss to follow-up

MAM: moderate acute malnutrition

MAZ: microbiota for age

MDCF: microbiota-directed complementary food

MDD: minimum dietary diversity

MUAC: mid upper arm circumference

NVD: normal vaginal delivery

RSV: respiratory syncytial virus

RTI: respiratory tract infection

SAM: severe acute malnutrition

SCFAs: short-chain fatty acids

SES: socioeconomic status

SGA: small for gestational age

WFAZ: Weight-for-age z score

WFHZ: weight-for-height z score

WHO: World Health Organization

Chapter 1: Background and aims

Background

Childhood malnutrition and its contribution to childhood mortality

The prevalence of malnutrition, specifically undernutrition, characterised by wasting (weight-for height < -2SD) or stunting (height-for-age < -2SD) has decreased over the past 20 years due to improvements in global food security and programs addressing risk factors for childhood malnutrition, but the global prevalence remains high. At current rates, no country will achieve the nutritional targets set by the World Health Assembly in 2012 (1). Stunting in children younger than 5 years improved globally between 2000 and 2017 (32.5% in 2000 versus 21.9% in 2017) (2). However, improvement in Africa has been the slowest, with a modest decline in prevalence from 38.8% to 30.0% during this period. Globally, wasting decreased from 10% to 7.3% between 2005 and 2017, with approximately a quarter of children affected by wasting residing in Africa (3).

Undernutrition significantly increases the risk of death, contributing either directly or indirectly to approximately 45% of deaths in children under 5 years (4). Data from longitudinal studies show that even moderate wasting and/or stunting increases that risk of mortality (RR = 2–3.4) with worsening mortality risk associated with the severity of wasting and/or stunting (RR = 4.8) (2). In a study evaluating cause of death using minimally invasive postmortem tissue sampling conducted in sub-Saharan African (including South Africa) and South Asia, the authors found that malnutrition was the most common underlying cause of death (104/632 [16.5%]) with pneumonia being one of the most common immediate causes of death (129/632 [24.8%]) (5).

At the opposite end of the malnutrition spectrum is overnutrition. Overnutrition and obesity are rising rapidly on a global scale with many low- and middle-income countries (LMICs) faced with the double burden of malnutrition (DBM) as undernutrition rates persist and overnutrition incidence rises (2). Data from the 2010s showed severe levels of DBM in more than one third (38%) of LMICs (based on stunting >30%, wasting >15%, and overweight >20%), with the highest levels in sub-Saharan Africa and South/East Asia (6). Historically, research on under- and overnutrition was conducted separately as these were considered distinct conditions. Data now show that the aetiology and pathogenesis of both conditions are driven by rapid shifts in living conditions and nutrition globally (7). Immediate factors such as diet or nutritional intake, including breastfeeding, timing and quality of complementary feeding, and the microbiome in early life play pivotal roles in the development of these conditions, potentially contributing to life-long and intergenerational

effects of malnutrition (7). Both under- and overnutrition negatively impact health throughout life. However, individuals are most susceptible to these negative impacts during early life (7). While preventative and therapeutic efforts have focused on undernutrition due to the significant negative short-term outcomes, overnutrition in early life has been shown to increase susceptibility to non-communicable disease with associated increased morbidity and mortality (7).

Determinants of malnutrition

The development of childhood malnutrition, both undernutrition and overnutrition, encompasses a complex interplay of factors including diet, food security (including access to quality foods), infection, environmental exposures, maternal physical and psychosocial health, and socioeconomic status (2,8). Studies have shown that caloric supplementation alone is often inadequate for correcting undernutrition, with only approximately 10% of children with stunting responding to nutritional supplementation (9). Additionally, recent research shows that the incidence of stunting and wasting are highest in the first 6 months of life, emphasising the importance of characterising and potentially intervening in the first 1000 days of life – from conception to 2 years of age (2). Unfortunately, wide scale environmental efforts to reverse malnutrition through improving hygiene, sanitation, and nutrition have shown limited effects, suggesting that current therapeutic options do not fully address disease pathogenesis (9–11). Data on factors associated with overweight and obesity in LMICs are limited (1).

The gut microbiome and malnutrition

Recently the gut microbiome has been identified as a determinant of malnutrition and as playing an important role in the development and persistence of both under- and overnutrition (7). The intestinal tract, specifically the large bowel, harbours the largest bioburden of microbes – predominantly bacteria – that function in a symbiotic relationship with the human host during periods of health (12). These commensal organisms contribute to a variety of metabolic pathways including vitamin metabolism, production of short-chain fatty acids (SCFAs) essential to gut health, bile acid metabolism, and harnessing nutrients from the diet (12–14). Importantly, the microbiome is pivotal for the maturation of a healthy immune system during childhood, determines response to vaccines and therapeutics, enhances colonisation resistance against invading enteric pathogens, and contributes to gut barrier integrity (13).

Maturation of the gut microbiome from birth through early childhood

The intrauterine environment during foetal development was originally thought to be sterile. However, studies have detected bacterial DNA in amniotic fluid, placental samples, and meconium

from newborns, suggesting in utero transmission of microbes and colonisation of the foetal intestinal tract (15). There is, however, consensus that the most significant period of bacterial colonisation of the infant's intestinal tract occurs at birth, with mode of delivery being an important determinant of the composition of the infant microbial community in the first few months of life. Children born vaginally are colonised by vaginal microbes such as *Lactobacillus*, whereas those born by caesarean section are colonised primarily by skin microbes such as *Staphylococcus* (15,16). Another key determinant of the infant microbiome is exposure to breast milk. Large birth cohorts have shown that exposure to breast milk, either exclusive or partial, was the most significant factor associated with community composition. Children exposed to breast milk had higher levels of *Bifidobacterium* species, and cessation of breastfeeding led to faster maturation of the gut microbiome (17). Following birth there is a predictable succession of bacterial communities, with the gut being dominated initially by facultative anaerobes (*E. coli*, Enterococcus, *Staphylococcus*, and *Streptococcus*) followed by obligate anaerobes (*Bifidobacterium*, *Bacteroides*, and *Clostridium*) (9). Microbial diversity increases with age with the most rapid increase occurring at the time of weaning, which often correlates with cessation of breastfeeding (17). By 3 years of age, the paediatric microbiome resembles that of an adult (18).

Other environmental exposures during childhood may affect the gut microbiome. Studies have evaluated the effect of antibiotic exposure on the developing microbiome during childhood with a recent systematic review and meta-analysis concluding that exposure to antibiotics reduced richness and diversity of the microbial community (19). Additionally, important community changes following antibiotic exposure included a reduction in beneficial taxa (*Bifidobacteria* and *Lactobacilli*) and increase in *E. coli* and *Bacteroidetes* (19). Macrolides were associated with greater disruption, with longer lasting effects on the microbial community. Other important factors known to affect the microbiome during early childhood include prematurity at birth, siblings, pet exposure, and daycare attendance (17,20,21).

Disruption of the microbiome increases the risk of infections such as childhood pneumonia and is associated with the development of chronic inflammatory disease (e.g., type 1 diabetes mellitus (DM), allergy, and autoimmune disease) (22–24). This increased risk of inflammatory/autoimmune disease is due to a dysregulated immune system secondary to microbial dysbiosis. Studies have shown a higher abundance of microbial genes associated with SCFA biosynthesis in control groups of children compared with those that developed type 1 DM (25). An in vitro study of bacteroides-derived lipopolysaccharide (LPS) from Finnish children at higher risk of T1D revealed reduced immune tolerance compared with *E. coli*-derived LPS from Russian children with low risk of T1D,

demonstrating the role of the microbiome in development of immune mediated disease (22). This study evaluated the gut microbiome longitudinally in 222 children. Early onset autoimmune disease was highly prevalent in Finnish and Estonian children and less so in the Russian children. Bacteroides species abundance was lower in Russian children compared to Finnish and Estonian children. Furthermore, Bacteroides derived LPS which was more prevalent in Finnish and Estonian children led to an exaggerated hyperinflammatory response compared to Russian children. These findings suggest that the microbial community composition during early life may play a role in predisposition to autoimmune disease. Children with asthma have been shown to be depleted in SCFA-producing genes compared with healthy, age matched controls and exhibit an immature microbial composition (26,27). In addition to the short term risks of a dysbiotic microbiome – such as malnutrition and infection risk – these findings further emphasise the importance of a healthy microbiome during early life and its impact on life-long disease risks.

Several cross-sectional studies have described differences in the composition of the microbial community in undernourished versus healthy controls. These studies were primarily conducted in children with severe acute malnutrition (SAM) with few studies in stunted populations (9). Emerging patterns of microbial dysbiosis in SAM across these studies include increased colonisation by enteric pathogens such as *E. coli*, *Shigella*, *Klebsiella*, and *Enterococcus*; reduced abundance of beneficial energy-harvesting genera such as *Bifidobacterium*, and reduction in beneficial anti-inflammatory, SCFA-producing microbes such as *Eubacterium*, *Lachnospiraceae* and *Ruminococcus* genera (9,28–30).

However, few studies have characterised the gut microbiome longitudinally in children with malnutrition. A landmark study by Subramanian et al. evaluated the microbiome in cohorts of undernourished and healthy children from Bangladesh and Malawi. Stool samples were obtained monthly from birth to two years (31). A microbiota-for-age z score (MAZ) was developed using a machine learning approach by identifying age-discriminatory bacterial species in children with consistently normal growth. This model, when applied to children with SAM and moderate acute malnutrition (MAM), showed that children with undernutrition exhibited a relative microbiota immaturity. Furthermore, standard-of-care nutritional interventions only partially resolved the gut microbial dysbiosis. This finding of low (immature) MAZ has been replicated in other studies of undernutrition (32,33), suggesting that microbial dysbiosis persists despite nutritional interventions and may play a role in children who do not respond to treatment. While the compositional characteristics of the microbiome are most frequently investigated, recent studies suggest that the functional metabolic potential of the microbiome community may play a similarly important role. In

a Zimbabwean cohort, functional metagenomic features were a better predictor of future growth than taxonomic composition (32).

Differentiating association versus causation in the context of the gut microbiome can be challenging. While there is much evidence demonstrating that the microbiome contributes to numerous metabolic pathways that affect the health of the host, the disease itself, including factors associated with disease, may alter the microbiome (e.g., antibiotic exposure, dietary changes). Microbiome interventional studies in human and animal models help disentangle cause from effect. Some have demonstrated development of the disease phenotype in germ-free mice following inoculation with gut bacteria from humans with the disease. While others show improvement of the disease itself with repair of gut microbiome dysbiosis by the administration of microbiome therapeutics in clinical trials. Several studies have shown the ability of the microbiome to transfer the undernourished phenotype by inoculating germ-free mice with gut bacteria derived from children with malnutrition (34,35).

Smith et al. demonstrated the causal role of the gut microbiome in the development of Kwashiorkor (oedematous malnutrition) by transplanting the gut microbiota from twins discordant for Kwashiorkor into germ-free mice (34). The combination of a Malawian diet and the Kwashiorkor microbiome led to the development of the malnourished phenotype in recipient mice. Blanton et al. transplanted gut microbiota from healthy and undernourished 6- and 18-month-old children from Malawi into germ-free mice, which resulted in the mice taking on the growth phenotype of their donor (35). Furthermore, transplanting the microbiota from the healthy mice to the undernourished mice resulted in resolution of growth deficits in the undernourished mice, primarily driven by the transfer of *Ruminococcus gnavus* and *Clostridium symbiosum* from healthy to undernourished mice. These findings not only implicate the microbiome in the causal pathway of malnutrition, but also highlight the potential of treating malnutrition with microbiome therapeutics. Further support for the potential of microbiome directed interventions was demonstrated in a positive interventional study of microbiota-directed complementary food (MDCF) in children with MAM, randomised to MDCF versus standard of care, showing significantly faster growth in the MDCF group (36).

There is also suggestive evidence that the microbiome is associated with the development of obesity. Animal models showed increased capacity of the obese mice microbiome to harvest energy from the diet, with the ability to transfer the obese phenotype through faecal microbiota transplantation (37). Clinical studies of faecal microbiota transplantation from lean donors into obese recipients showed improvement in metabolic markers (38). Despite the rapidly rising prevalence of obesity in children

globally, there are few studies of the microbiome in childhood obesity and further research is needed to understand the role of the microbiome in obesity development.

Overall, the evidence suggests that the microbiome established in early childhood has an important role in the development of both under- and overnutrition. Strategies to optimise the development of a healthy microbiome during early childhood may be important to promote health.

Malnutrition and childhood pneumonia

Pneumonia remains a leading cause of death in children under 5 years. While substantial progress has been made in the reduction of under-5 mortality globally, levels remain far above the United Nations Sustainable Development Goals. In 2019, approximately 5.3 million deaths occurred in children under-5 globally with 672 000 childhood pneumonia deaths occurring in LMICs (39,40). Although the global under-5 mortality rate has decreased over the past two decades, children in sub-Saharan Africa remain the most vulnerable, with a 14 times higher risk of death during childhood, compared with Europe and North America (41).

Aligned with the decrease in pneumonia-associated mortality in children under-5 globally, the incidence of pneumonia and severe pneumonia decreased by 30% in this age group between 2000 and 2015 (42). However, hospital admissions due to child pneumonia have increased by 187% during this period, especially in LMICs (42). Increase in incidence of hospital admission was most prevalent in south Asia and may be driven by improved access to healthcare facilities and health-seeking behaviour. The overall decrease in incidence in pneumonia is attributable to the decline in the major risk factors for childhood pneumonia, including a 45% decrease in pneumonia episodes in children living with children with HIV; a decrease in malnutrition incidence globally (7–72% depending on region); a 5% decrease in incomplete immunisations, and improved expanded childhood immunisations. Pneumococcal pneumonia was the leading cause of pneumonia related morbidity and mortality. Expanded vaccines coverage directed against respiratory pathogens such as *Haemophilus influenza* B and *Pneumococcal* pneumonia have led to a 5% and 8% decrease respectively in respiratory tract infection related mortality in children under 5 years globally (43). However, an increase in some pneumonia risk factors such as non-exclusive breastfeeding, indoor air pollution, and low birth weight (LBW) were observed between 2000 and 2015 (42). In South Africa, there were approximately 320 000 pneumonia episodes and 4100 pneumonia-related deaths in children under 5 in 2017, with a decline in incidence of approximately 50% between 2000 and 2015 (42,44). This decline in incidence is partially attributable to a significant decline in childhood HIV infection (approximately 70%). Despite the general decline in local and global incidence, childhood pneumonia remains the leading cause of morbidity in children under 5, especially in LMICs,

accounting for 50% of sick child healthcare consultations (45). Of note, this decline in pneumonia incidence was observed prior to the COVID-19 pandemic with evidence showing rebound hospitalisation rates to pre-pandemic levels in children under 5 (46). Understanding the epidemiology and risk factors for disease facilitates the development of novel preventative, diagnostic and therapeutic strategies.

Risk factors for the development of childhood pneumonia

Targeting the risk factors associated with the development of pneumonia is a key strategy for reducing both pneumonia incidence and mortality. In LMICs, several additional risk factors are associated with childhood pneumonia, including HIV exposed-uninfected infants, young maternal age, low maternal education, and low socioeconomic status (45). Age remains an important risk factor for disease severity and mortality with one systematic review showing a 2.5-fold higher risk of death in children under 1 year compared with those aged 1–5 years (47).

Malnutrition is one of the most important risk factors for both development of and mortality from childhood pneumonia (43). Specifically, wasting was found to be the leading risk factor for mortality in children with pneumonia, responsible for 61.4% of pneumonia-related deaths in 2016 (43). The risk of death from childhood pneumonia has been shown to correlate with the severity of malnutrition. A large case series including over 5000 children under 5 years, hospitalised with severe pneumonia, showed that severe under-weight (weight-for-age z-score <3) was significantly associated with death during the pneumonia episode (48). Kirolos and colleagues conducted a systematic review and meta-analysis that included over 33 000 children with pneumonia-related deaths from 23 studies that measured nutritional status between 1980 to 2020 (49). The authors showed that the estimated odds ratio (OR) of pneumonia-related death was 2.0 (95% CI 1.6–2.6) for moderately underweight children and 4.6 (95% CI 3.7–5.9) for those severely underweight (49). Most studies reported data on weight-for-age, whereas data on height-for-age (stunting) were limited with only 6 studies measuring height, showing a higher OR of death in stunted children (OR 2.7 CI 0.8–12.5). Additionally, the authors found that despite reductions in overall incidence and mortality from childhood pneumonia over the past two decades, there has been little improvement in the risk of death in malnourished children with pneumonia.

Several studies have demonstrated a correlation between stunting and increased risk of death in children with pneumonia (47,49). A systematic review and meta-analysis determining the risk factors for mortality from childhood pneumonia found a twofold increase in the risk of death for children

with moderate stunting (47). Similarly, stunting increased the risk of developing childhood pneumonia and severity of disease (50–52). A previous analysis of determinates of pneumonia in a well-phenotyped South African birth cohort, the Drakenstein Child Health study (DCHS) found that severe stunting was positively associated with incidence of pneumonia (IRR 1·76 [1·17–2·58]) (53). In terms of obesity, the opposite end of the malnutrition spectrum, there is a currently no evidence to suggest that obesity or overweight is a risk factor predisposing to pneumonia during early childhood. However, current data in children under 5 years remains sparse (54).

Malnutrition plays a complex role in LMICs as both an immediate cause of death in children and as a major risk factor for susceptibility and mortality from childhood pneumonia. Malnutrition results in impaired innate and adaptive immune function, increasing susceptibility to respiratory tract infections (55). This form of secondary immune dysfunction has been poorly characterised (56).

The role of the microbiome in development of pneumonia

The gut–lung axis refers to the connection between the gut microbiome, lung microbiome, and lung immunity or susceptibility to respiratory tract infection (23,24,57). This is a bidirectional system in which gut microbial metabolites influence lung susceptibility to infection, mediated by the immune system, and where lung infection results in changes to the composition of the gut microbiome. There are several mechanisms by which the gut microbiome exerts a protective effect against viral lung infections, mostly shown in animal models. Microbial metabolites such as desaminotyrosine, produced by *Clostridia*, enter circulation and prime the immune system to protect against influenza virus by the upregulation of type 1 interferon (IFN) production (58). SCFAs, produced by numerous gut bacteria including members of the *Lachnospiraceae* family, have been shown to similarly enter systemic circulation, leading to upregulation of type 1 IFN via G-protein coupled receptors. SCFAs increase lung macrophage production and enhance CD8 T-cell effector function, increasing their ability to kill virus-infected cells, thereby protecting against viral respiratory tract infections (RTI) (23). Additionally, bacterially-derived toll-like receptor ligands have been shown to play a critical role in defence against viral RTI (23). A healthy gut microbiome primes the adaptive immune system against RTI by enhancing CD8 T cell function (24). Interventional studies in mice using *Lactobacillus johnsonii* supplementation reduced severity of respiratory syncytial virus (RSV) infection (59).

A limited number of cross-sectional studies have demonstrated compositional differences in the gut microbiome between children with pneumonia and healthy controls. However, no longitudinal studies have characterised the gut microbiome during pneumonia episodes. A U.S. study of hospitalised infants with bronchiolitis compared with healthy age-matched controls showed a higher

risk of bronchiolitis in children with Bacteroides-dominant profiles (60). Another U.S. case-control study of infants admitted to hospital with RSV bronchiolitis showed enrichment in S24_7, *Clostridiales, Odoribacteraceae, Lactobacillaceae*, and *Actinomyces* in RSV cases versus healthy controls. Alpha diversity was decreased in cases compared to controls and a significant difference in beta diversity was found between the groups (61). Based on these limited studies, the gut microbiome in children appears to be dysbiotic during pneumonia episodes when compared with healthy controls. However, taxonomic composition appears to vary between studies. Additionally, none of these studies were conducted in LMICs, where the burden of pneumonia is highest and data may inform future strategies to reduce pneumonia incidence.

Findings in animal and clinical studies show that RTIs can affect the composition of the gut microbiome through several mechanisms (23). Increase in inflammatory cytokines such as type 1 and interferon gamma have been shown to alter the gut microbiome composition, favouring the proliferation of Proteobacteria in mice models. Other factors that may contribute to changes in the gut microbiome include reduced appetite during infection, intestinal inflammation, and reduced colonisation resistance (23,57).

The interplay of early life gut microbiome disruption, malnutrition, and childhood pneumonia

Strategies aimed at reducing the prevalence of pneumonia and malnutrition may have reached a therapeutic ceiling. Several studies aimed at improving malnutrition using large scale interventions including improvements in access to therapeutic feeds, clean water, sanitation, and hygiene did not improve nutrition outcomes (32).

Evidence of the significant contribution of the gut microbiome to health during infancy, childhood, and subsequent adult life is now well established. Disruption of the gut microbiome during early childhood may be an important and under recognised risk factor for both malnutrition and pneumonia and, hence, an important contributor to under-5 mortality in children.

Several risk factors are common to the microbiome, malnutrition, and childhood pneumonia. The duration of EBF is a well-described determinant of gut microbial composition, and a well-established risk factor for the development of malnutrition or pneumonia during childhood. EBF is known to increase the abundance of the beneficial *Bifidobactrium* genus, which enhances digestion of human milk oligosaccharides (HMOs) found in breastmilk and has been found to be depleted in infants with acute malnutrition (28). Furthermore, treatment with *B. infantis* and HMO improved weight gain in infants with SAM. Several studies have also shown that *Bifidobacterium* is reduced in children with pneumonia compared with healthy controls, suggesting a protective role of *Bifidobactrium* in

childhood pneumonia (24,62). This may be mediated through the immune system, with studies showing that *Bifidobactrium* contributes to immune maturation during early life.

LBW is another important determinant of the early life microbiome composition, risk of malnutrition, and pneumonia (2,20). LBW due to prematurity results in an immature microbial community, dominated by gammaproteobacteria (20). The 'immature' preterm microbiome may play a role in the impaired growth and increased risk of infections associated with LBW and prematurity. Grier et al. showed that delayed maturation of the preterm infant's microbiome is associated with growth failure (63).

While the two risk factors outlined above are well known and common to both malnutrition and pneumonia, further studies are required to determine whether other microbiome-modifying exposures are associated with increased risks of malnutrition and pneumonia. Such exposures include broad spectrum antibiotic exposure during early life, maternal microbiome, diet, and household exposures (pets and siblings). Clearly, there is a complex interplay of risk factors for pneumonia and malnutrition with microbial dysbiosis playing a role in the pathogenesis of both. Further research should focus on children in LMICs – especially in Africa – given the burden of disease in these settings and the current paucity of data on the microbiome in children residing in sub-Saharan Africa. Ideally, these studies should encompass longitudinal microbiome sampling in children with malnutrition and childhood pneumonia. Identifying modifiable risk factors, such as gut microbiome disruption, that are known to contribute to both pneumonia and malnutrition susceptibility may be highly beneficial and lead to the development of novel therapeutics to reduce the incidence and mortality associated with these childhood diseases.

To address these gaps, a prospective study was undertaken of children enrolled in the DCHS, a South African birth cohort study. The overall aim was to investigate determinants of growth from birth through early childhood, and the association of the intestinal microbiome with growth and pneumonia in an LMIC context.

Aims and hypotheses

The overall aim of this thesis was to investigate determinants of growth in early childhood, and the association of the intestinal microbiome with growth and pneumonia in an LMIC context.

Hypotheses

1. Poor infant growth (stunting, wasting, under-weight for age) is substantially prevalent in low socioeconomic populations in South Africa.

2. There are modifiable risk factors that contribute to the development of poor growth during infancy.
3. The choice of infant milk feeds (breast milk versus infant formula) and quality of complementary feeds is associated with growth outcomes at 1 year.
4. Poor linear growth is associated with disruption (dysbiosis) of the gut microbiome in early life.
5. The gut microbiome is dysbiotic during pneumonia episodes in children.

Specific objectives

1. To investigate infant growth and risk factors for poor growth in the first year of life.
2. To describe infant feeding practices and their association with growth outcomes at 12 months.
3. To investigate the association between linear growth and the microbiome in children residing in an LMIC.
4. To investigate the gut microbiome of young children with pneumonia in an LMIC.

Research setting

This study was nested within the ongoing DCHS, a birth cohort study following more than 1000 mother–child pairs through pregnancy, birth, and early childhood. The study is in a low income, peri-urban setting near Cape Town, South Africa. A primary aim of the DCHS was to examine the incidence, aetiology and outcome of childhood pneumonia during the first 5 years of life. The comprehensive collection of pneumonia risk factors in the DCHS including detailed nutritional data (food frequency questionnaires, longitudinal anthropometric measurements), biomarkers including frequent stool collection, immunological data (HIV exposure), environmental exposure, maternal health and psychosocial data (socio-economic status, food security) enabled comprehensive analysis of risk factors related to nutrition, growth, the gut microbiome, and pneumonia.

Ethical considerations

The study was approved by the University of Cape Town Faculty of Health Sciences Human Research Ethics Committee (HREC 739/2016). Mothers provided written informed consent at enrolment, with annual renewal.

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Chapter 2: Maternal and infant factors had a significant impact on birth weight and longitudinal growth in a South African birth cohort

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ABSTRACT

Aim: This birth cohort study investigated longitudinal infant growth and associated factors in a multi-ethnic population living in a low-resource district surrounding the town of Paarl in South Africa.

Methods

Between March 2012 and October 2014, all mothers attending their second trimester antenatal visit at Paarl Hospital were approached for enrollment. Mother-infant pairs were followed from birth until 12 months of age. Comprehensive socio-demographic, nutritional and psychosocial data were collected at birth, two, six and 12 months. Infant anthropometry was analysed as z-scores for weight and height. Linear regression was used to investigate predictors of birth weight and linear mixed effects models were used to investigate predictors of infant growth.

Results

Longitudinal anthropometric data from 792 infants were included: 53% were Black African, 47% were mixed race and 15% were born preterm. Stunting occurred in 13% of infants at 12 months. Maternal height, antenatal alcohol and tobacco use, ethnicity and socioeconomic status were significant predictors of birth weight. In the adjusted mixed effects model, birth weight was a significant predictor of growth during the first year of life.

Conclusion

Birth weight was an important predictor of growth trajectory during infancy. Birth weight and growth were influenced by several important modifiable factors.

KEY NOTES:

This large cohort study investigated the factors associated with birth weight and longitudinal infant growth in a resource-poor setting.

Birth weight was a major predictor of growth trajectory during infancy, after adjusting for socioeconomic status, ethnicity, prematurity and intrauterine growth restriction.

Alcohol or tobacco consumption during pregnancy was high in this population and was associated with both poor birth outcomes and poor post-natal growth during infancy.

INTRODUCTION

Childhood malnutrition, which encompasses both under-nutrition and overweight or obesity, is highly prevalent in low and middle-income countries (LMIC) (1). Although global rates of stunting in children under five years of age have decreased over the past decades, the prevalence of stunting in sub-Saharan Africa has continued to rise (1). Obesity rates in children and adults in LMIC have also been rising and these are referred to as the double burden of malnutrition (1,2).

South Africa comprises multi-ethnic populations, with varying socioeconomic circumstances, psychosocial factors, disease exposures and cultural practices. Differences in child growth patterns may occur in such population groups and these reflect genetic or environmental factors or a combination of these (3,4). The prevalence of psychosocial factors, such as maternal depression, intimate partner violence (IPV) and substance abuse – including alcohol and tobacco consumption - in LMIC are high and have been associated with poor infant outcomes, including low birth weight and poor infant growth (5).

Few cohort studies from LMIC have investigated child growth longitudinally and no longitudinal studies of growth patterns in South African infants from different ethnic backgrounds has been carried out. The aim of this study was to describe patterns of growth and factors that had an impact on growth in South African infants enrolled in the Drakenstein Child Health Study.

PATIENTS AND METHODS

The primary aim of the Drakenstein Child Health Study birth cohort was to investigate the epidemiology, risk factors, aetiology and long-term impact of respiratory illness on child health, including investigations into growth and nutrition in early childhood (6).

Study population

Pregnant women living in a resource poor area surrounding an agricultural town, were enrolled during their second trimester of pregnancy at two primary healthcare facilities serving different ethnic communities: TC Newman serving a mixed race community and Mbekweni serving a Black African community. This means that the two clinics were synonymous with ethnicity. Women who were less than 18 years of age, those who were unable to provide informed consent or those who intended to leave the area within a year were excluded. All the infants that were born to mothers enrolled into the study were included in the analysis. All pregnant women who were enrolled into the study received standard antenatal care at a single district level hospital.

Measures

Socio-demographic and health information were obtained from questionnaires administered to the mothers during antenatal and postnatal study visits. Four sociodemographic variables were used to generate a relative socioeconomic score (SES): education, employment, household income and assets. Participants were stratified into quartiles based on their SES score. These quartiles were labelled as low, low-to-moderate, moderate-to-high or high SES, to enable comparisons within the study population (7).

All births occurred at a single public hospital, Paarl hospital, which provides general hospital and emergency care for a population of more than 600,000 people living in a vast geographical area of approximately 22,500 square kilometres. It is based in a predominantly semi-rural region 60 kilometres from Cape Town in the Southern tip of South Africa.

Birth weight and length were measured at the hospital. Gestational age at birth was estimated based on an antenatal ultrasound carried out in the second trimester (68%) and if this was unavailable then fundal height (28%) or maternal recall of the last menstrual period (4%) was used. Trained study staff collected serial anthropometric measurements on the infants at two, six and 12 months. The infant's length was measured in centimeters to the nearest completed 0.5cm and their weight was measured in kilograms to the nearest completed 10g, according to standard operating procedures. Maternal height was measured to the nearest 0.1cm at enrollment, using a wall-mounted stadiometer. The birth anthropometrical measurements were conducted by trained labour ward staff and the birth weights and lengths were obtained from the patients' folders. When we compared the labour ward staff measurements of infant anthropometry with study staff measurements in a subgroup of patients, we found no significant differences.

Weight was measured, in light or no clothing, using the TAN1584 digital platform scale (Tanita, Illinois, USA) and recumbent length using the seca210 length-measuring mat (seca, Hamburg, Germany). The latter was performed on a firm surface by two staff members. The equipment was calibrated weekly. All anthropometric measurements were carried out twice for each child, with a third measurement if the difference between the first and second measurement differed by more than 0.5cm for length or more than 0.5kg for weight. Study staff underwent regular anthropometric training and assessment every three months and this was conducted by the lead anthropometrist.

Four time-points were used for the purposes of this analysis: birth, two, six and 12 months of age. A valid anthropometric measurement was within four weeks before or after that time point.

With regard to nutritional information, the infant feeding practice was categorised based on the World Health Organization's (WHO) Infant and Young Child feeding indicators, namely exclusive breastfeeding or bottle feeding, which included infant cows' milk-based formula with or without breast milk or semi solids (8).

Validated questionnaires were administered to mothers antenatally and six weeks postpartum to assess psychosocial factors (5). The Edinburgh Postnatal Depression Rating Scale was used to assess maternal depression and a score of at least 13 was classified as probable depression (5). An IPV questionnaire was used to assess recent exposure to physical, emotional and sexual IPV (5). Exposure was categorised as above the threshold if a participant reported more than one incident of IPV during the previous year. The Alcohol, Smoking and Substance Involvement Screening test was used to assess maternal alcohol consumption based on a scoring system that was previously described (5). Tobacco smoking was measured using maternal urinary cotinine levels antenatally and postnatally using the IMMULITE 1000 Nicotine Metabolite Kit (Siemens Medical Solutions Diagnostics, Caernarfon, United Kingdom). A urinary cotinine level of ≥500 ng/ml was used as the threshold to dichotomise mothers into active smokers and non-smokers based on the manufacturer's recommendations.

Statistical analysis

Birth weight and length were converted to z-scores for gender and gestational age using the INTERGROWTH-21st standards (9). Infants were classified into low birth weight (LBW) if they weighed less than 2,500g at birth, preterm if they born at less than 37 weeks of gestational age and small for gestational age (SGA) if they weighted less than the 10^{th} percentile for gestational age, based on gender and gestational age. Corrected gestational age was used to calculate the WHO z-score of preterm infants.

The weight and length measurements following birth were converted to z-scores based on age and gender, using Anthro software (WHO, Geneva, Switzerland) (10). Weight-for-age z-scores (WFAZ), height-for-age z-scores (HFAZ), weight-for-height z-scores (WFHZ) and body mass index (BMI) for age z scores (BMIZ) were calculated. BMI was calculated using the formula weight in kilograms divided by length in centimetres squared.

Growth patterns in the study population were analysed at each time point by calculating the median WFAZ and HFAZ as well as between the time points by calculating the difference in WFAZ and HFAZ. At each time point, indicators of malnutrition were calculated so that infants with an HFAZ <-2 were classified as stunted, those with a WFAZ <-2 as under-weight-for-age, those with a WFHZ <-2 as wasted and those with a BMI for age >2 as overweight and >3 as obese. Growth patterns and

indicators of malnutrition were stratified by study site, in other words ethnicity. Significant differences in WFAZ and HFAZ, indicators of malnutrition, and changes in WFAZ and HFAZ between time points were identified using chi-square or Fisher's exact tests for categorical variables and *t*-tests or Wilcoxon rank sum tests (Mann-Whitney tests) for continuous variables.

Variables significantly associated with infant WFAZ at birth were explored. Two linear regression models were built to investigate maternal height and antenatal smoking, based on urinary cotinine levels, as independent predictors of *a priori* interest. For all models, regression coefficients with 95% confidence intervals (95% CIs) were calculated to determine the strength of these associations.

Based on the literature, known variables associated with infant growth during the first year of life were explored using linear mixed effects models. Ethnicity and SES were included as fixed effects and WFAZ at birth and age at time of study visit were included as random effects. Three models were built. The first adjusted model (model one) included all variables that were significant in the unadjusted model. The final model (model two) only included the variables that remained significant in model one.

The statistical analysis was conducted using Stata 12 (StataCorp Inc, College Station, Texas, USA).

Ethical approval was obtained from the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee and the Provincial Child Health Research committee. Written informed consent was obtained from all the participants.

RESULTS

From March 2012 to October 2014, we enrolled 789 mothers with a median age of 25.7 (21.8-30.7) years who had given birth to 792 infants, including three pairs of twins (Table 1) and all of these infants completed their birth study visit. At the time of analysis, 681 infants (93%) completed their two-month study visit, 517 (71%) completed their six-month study visit and 342 (47%) completed their 12-month study visit (Figure 1). We excluded 62 infants from the growth analysis due to incomplete data: 35 infants at two months and 27 infants at six months. In addition, 97 mother-infant pairs were lost to follow up during the course of the study. The mothers who were lost to follow up were significantly younger than those who remained in the study (median age 23.3 years versus 26.0 years; p=0.003), had higher levels of education (p=0.030) and were more likely to be primigravid (p=0.048). There were no significant differences in birth characteristics between the infants who were lost to follow up and those who remained.

The study population had a predominantly low income, with 43% of households earning below the minimum wage of 5,000 South African Rands per month (11). Only half of the participants in the highest SES quartile reported a household income that was higher than that level. Just over a third (34%) of the Black African households fell into the lowest SES quartile and they were relatively poorer than mixed race households (Table 1). The reported levels of antenatal substance use were significantly higher in mothers of mixed race infants than Black African infants. More than half of the mixed race mothers were active smokers (51%) based on their antenatal urinary cotinine levels and more than a quarter (28%) reported consuming alcohol antenatally and postnatally. More than a quarter of mothers (26%) were classified as experiencing probable post-natal depression. Recent IPV was significantly higher among mixed race mothers than Black African mothers.

The median gestational age at birth was 39 weeks, but 15% of babies were born preterm (Table 1). More than a quarter of infants (26%) were born SGA while 15% were LBW (Table 1). The median WFAZ at birth was below the WHO reference population and this was more marked in mixed race babies (Table 2). Of the 119 infants born preterm, 87 were late preterm (34-37 weeks), 20 were moderately preterm (32-34 weeks) and 12 were very preterm (<32 weeks). The median (IQR) gestation of the preterm infants was 35 (33-36) weeks. The median (IQR) birthweight of the preterm infants was 2.5 (2.05-2.88) kg. In total, 106 of the preterm infants were appropriate for gestational age (AGA), and 13 were SGA. The median WFAZ improved from birth to two months, with a median change of 0.2 between birth and two months. Between two and six months, infants exhibited higher weight gain than height gain. At six months, Black African infants were, on average, heavier and taller than mixed race infants. The period of highest weight gain during infancy was between birth and six months, with a difference in WFAZ of 0.7 (Table 2). At 12 months, the median WFAZ of the sample was almost identical to the WHO median, with a slowing up of weight gain between six and 12 months. Median HFAZ remained negative at all time points, indicating that the infants had slower linear growth and remained shorter than infants from the reference WHO population (Table 2). On average, children born to Black African mothers weighed more and were taller at birth and remained heavier and taller at each time point than those born to mixed race mothers (Table 2).

Malnutrition, either under or over nutrition, occurred consistently throughout infancy (Table 3). Stunting was the most prevalent indicator of under-nutrition at all time points, ranging from 17% of infants at two months to 13% by 12 months. At 12 months of age 2% of the children were obese and 6% were overweight and the rates were higher among black African than mixed race infants (Table 3)

In the unadjusted models of infant WFAZ at birth, ethnicity, household income, gravidity, maternal alcohol and tobacco use, recent IPV and maternal height were significantly associated with lower WFAZ at birth (Table 4). Only variables that were significant in the unadjusted models were included into the multivariate model. Psychosocial and nutritional models were built separately. In the psychosocial multivariate model, the associations between both maternal alcohol and tobacco use and lower WFAZ at birth remained significant, independent of ethnicity and SES (Table 4). In the nutritional model, increased maternal height, which was used as a proxy for maternal nutritional status, remained significantly associated with higher infant WFAZ at birth, when adjusted for ethnicity and SES (Table 4).

In the univariate analysis, birth weight, ethnicity, SES, antenatal and postnatal IPV and antenatal and postnatal maternal substance use - specifically alcohol or tobacco - were significantly associated with infant growth, as measured by repeated WFAZ measurements over the first year of life (Table 5). However, only birth weight and antenatal and postnatal maternal substance use remained significantly associated with infant growth after controlling for ethnicity and SES. In the final multivariate model, birth weight, ethnicity, SES and antenatal maternal substance use remained significantly associated with growth during infancy, adjusted for infant age at the time of measurement. Infant WFAZ at birth was an important predictor of WFAZ throughout the first year, independent of ethnicity, SES and antenatal maternal substance use.

DISCUSSION

In this South African birth cohort, we found a high prevalence of LBW and SGA infants and a mean WFAZ at birth below the WHO reference population. Despite some catch-up growth, a substantial prevalence of childhood stunting was observed in this sample. Black African children had better nutritional status from birth that persisted throughout infancy compared to mixed race infants. We also demonstrated an emerging group of over-weight and obese infants, with a higher prevalence among black African infants. Antenatal maternal smoking and alcohol consumption and maternal height were important independent predictors of infant birth weight. Birth weight, SES, ethnicity and maternal antenatal alcohol and tobacco consumption were important determinants of the infant's longitudinal growth during the first year of life.

Our results were consistent with studies that showed that birth weight was a strong determinant of longitudinal infant growth in the first year (1). In our sample, factors such as maternal smoking and alcohol consumption during pregnancy, gravidity and poor maternal nutrition were strong predictors of a lower weight at birth (Table 5) and were consistent with findings from other studies (1,12–14).

LBW is a significant risk factor for poor growth during infancy and may be due to prematurity, being born SGA or a combination of these factors (1). The prevalence of LBW infants in this study was higher than the figures reported by other African and South African studies (15,16). Our findings that birth weight was a predictor of growth during infancy may be due to the high prevalence of LBW and SGA infants in this population. However infant birth weight was still a significant independent predictor of infant growth during the first year in the subgroup analysis, when infants with LBW or SGA were excluded ($p < 0.001$) (Table 6). As demonstrated in other studies, infants born term SGA and preterm SGA had significantly slower growth than those born preterm and term AGA (Figure 2) (17–19). Only 10% of the preterm infants were early preterm, which is a very small proportion.

We demonstrated the independent negative effects that smoking and alcohol consumption during pregnancy had on both birth weight and postnatal infant growth, which persisted after controlling for SES and ethnicity. This may have been partially mediated through the effects of alcohol and tobacco on fetal growth (14,20). However, further investigations into the mechanistic effects on postnatal growth are required, for example the effects of these substances on infant micronutrient status, fetal programming, the intestinal microbiome and epigenetic alterations (21). Similar effects of antenatal tobacco or alcohol exposure on infant growth have been shown in other studies (14,22).

SES was an important determinant of infant growth. Consistent with other studies in LMIC, higher SES promoted infant growth (23). This may have been due to better maternal health, better nutritional knowledge, being more able to afford formula or complementary feeding and better health seeking behaviour (24). Ethnic disparities in infant health have been described in several multi-ethnic countries globally and have mainly resulted from differences in maternal health, access to healthcare services and SES (4). Unexpectedly, black African infants had higher birth weights and better longitudinal growth than mixed race infants, despite coming from lower SES households, and these effects persisted after controlling for birth weight and SES. Additional genetic or environmental interactions may be important in determining growth during infancy and these require further investigation (1).

We found a high prevalence of stunting in this sample, particularly at two months of age, with a marginal decline by 12 months of age. These results were consistent with cross-sectional South African studies that have reported a similar high prevalence of stunting in children (25). The high prevalence of stunting is surprising given the study setting, with access to healthcare facilities, free maternal antenatal care, nutritional programmes and a strong primary healthcare programme. SES, maternal, nutritional, genetic, or infant factors, such as environmental enteric dysfunction may have been contributory factors and further studies of these are underway.

In contrast, we also found a concerning prevalence of overweight or obese infants. To the best of our knowledge, no previous studies have documented the prevalence of overweight and obese infants in South Africa. These findings may reflect changes in lifestyle, diet or poor feeding practices (26) and a detailed analysis of such factors is currently being undertaken. A previous study within this population showed no correlation between birth weight and overweight or obesity at 12 months of age (27).

The strengths of this study included the prospective design with good cohort retention, the longitudinal measurements of multiple parameters of infant growth by trained study staff, the inclusion of two ethnically distinct South African population groups and the collection of comprehensive SES, maternal and nutritional information. In addition, using mixed effects modelling of growth enabled us to model individual growth trajectories, which are by nature comprised of repeated, dependent measurements over time, in a single model. This added to the quality of our results. Some limitations also need to be noted and these were that these findings may not be generalisable to other African or LMIC populations. However, many of the factors explored in this analysis are highly prevalent in LMIC.

This was the first study of this magnitude to evaluate longitudinal growth in a developing county during the first year of life, including multiple maternal, infant, psychosocial and environmental risk factors that have been associated with poor infant growth. It highlights some of the adversities that children in developing countries face, which arise as early as intrauterine life.

CONCLUSION

Our findings of the predictive nature of birth weight on growth trajectory during infancy emphasise the importance of maternal health and antenatal care on infant growth. Simple, cost-effective interventions that focus on maternal health, such as programmes to reduce alcohol consumption and smoking and improve nutrition, may improve birth weights and subsequent postnatal growth, thereby promoting child health. Further studies are required to investigate environmental and genetic determinants of infant growth in LMIC.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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Table 1 Maternal demographic and psychosocial characteristics and infant birth outcomes

Variable	Mbekweni (Black African)– *n* (%)	TC Newman (Mixed race)– *n* (%)	Total – *n* (%)	*p*-value
Maternal demographic and psychosocial characteristics				
Number of mothers	418 (53)	371 (47)	789	
Median [IQR] gestation at enrolment	23 [20-26]	21 [19-25]	22 [20-25]	<0.001
Ethnicity				
Black/African	414 (99)	5 (1)	419 (53)	
Mixed race	4 (1)	363 (98)	367 (47)	
Other	0 (0)	3 (0.8)	3 (0.4)	
Median [IQR] age at enrolment (yrs)	26.7 [22.2-31.8]	24.8 [21.3-29.1]	25.7 [21.8-30.7]	<0.001
Median [IQR] height (cm)	160 [156-164]	158 [153-162]	159 [155-163]	<0.001
Educational achievement				
Primary education only	42 (10)	29 (8)	71 (9)	
Some secondary education	225 (54)	182 (49)	407 (52)	
Completed secondary ed.	122 (29)	144 (39)	266 (34)	0.021
Tertiary education	29 (7)	16 (4)	45 (6)	
Antenatal urine cotinine – active smoker	58 (14)	190 (51)	248 (31)	<0.001
Antenatal self-reported alcohol use	34 (8)	105 (28)	139 (18)	<0.001
Antenatal depression – above threshold (EPDS)	114 (27)	90 (24)	204 (26)	0.335
Recent intimate partner violence (antenatal) – above threshold	110 (26)	146 (39)	256 (32)	<0.001
Primagravida	134 (32)	146 (39)	280 (35)	0.033
HIV-infected	158 (38)	10 (3)	168 (21)	<0.001
Unemployed	327 (78)	260 (70)	587 (74)	0.009
Average household income				
<R1000 (<$84) /month	203 (49)	127 (34)	330 (42)	
R1000-R5000($84-$420) /month	167 (40)	174 (47)	341 (43)	<0.001
>R5000 (>$420)/month	48 (11)	70 (19)	118 (15)	
SES quartile				
Lowest SES	144 (34)	69 (19)	213 (27)	
Low-moderate SES	109 (26)	88 (24)	197 (25)	<0.001
Moderate-high SES	99 (24)	94 (25)	193 (24)	
Highest SES	66 (16)	120 (32)	186 (24)	
Early postpartum maternal psychosocial characteristics (n=366)				
Self-reported tobacco use	6 (3)	91 (54)	97 (27)	<0.001
Self-reported alcohol use	10 (5)	44 (26)	54 (15)	<0.001
Depression – above threshold (EPDS)	47 (24)	29 (17)	76 (21)	0.115
Recent intimate partner violence – above threshold	42 (21)	47 (28)	89 (24)	0.149

Infant birth outcomes				
Number of infants	421 (53)	371 (47)	792	
Infant sex				
Female	210 (50)	164 (44)	374 (47)	
Median [IQR] gestation at delivery (weeks)	39 [38-40]	39 [37-40]	39 [38-40]	0.360
Pre-term birth (<37 weeks)	64 (15)	55 (15)	119 (15)	0.882
Low birth weight (<2500g)	45 (11)	71 (19)	116 (15)	0.001
Small for gestational-age	96 (23)	107 (29)	203 (26)	0.052

Table 2 Infant anthropometry and feeding

Variable[a]	Mbekweni (Black African)	TC Newman (Mixed Race)	Total	*P*-value
Birth, (n)	421	371	792	
Median WFAZ [IQR]	-0.4 [-1.2-0.3]	-0.7 [-1.4 - -0.1]	-0.5 [-1.3-0.1]	<0.001
Median HFAZ [IQR]	0.1 [-0.8-1.1]	0.003 [0.8-0.9]	0.1 [-0.8-1.0]	0.019
2 months, (n)	344	337	681	
Median WFAZ [IQR]	-0.1 [-0.9-0.6]	-0.5 [-1.4-0.2]	-0.3 [-1.1-0.4]	<0.001
Median HFAZ [IQR]	-0.5 [-1.3-0.5]	-0.9 [-1.9-0.0]	-0.7 [-1.6-0.2]	<0.001
Median change in WFAZ between birth and 2 months [IQR] (*n*=681)	0.3 [-0.5-1.0]	0.1 [-0.5-0.7]	0.2 [-0.5-0.9]	0.017
Median change in HFAZ between birth and 2 months [IQR] (*n*=681)	-0.7 [-1.6-0.3]	-1.0 [-1.8-0.1]	-0.9 [-1.7-0.2]	0.040
6 months, (n)	243	274	517	
Median WFAZ [IQR]	0.5 [-0.4-1.2]	-0.05 [-1.0-0.8]	0.2 [-0.7-1.0]	<0.001
Median HFAZ [IQR]	-0.2 [-1.3-0.7]	-0.7 [-1.6-0.2]	-0.4 [-1.5-0.5]	0.002
Median change in WFAZ between 2 and 6 months [IQR] (*n*=500)	0.5 [-0.1-1.2]	0.4 [-0.1-1.0]	0.4 [-0.1-1.1]	0.455
Median change in HFAZ between 2 and 6 months [IQR] (*n*=500)	0.3 [-0.8-1.3]	0.2 [-0.5-0.9]	0.2 [-0.6-1.1]	0.732
12 months, (n)	155	187	342	
Median WFAZ [IQR]	0.3 [-0.5-1.0]	-0.1 [-1.1-0.6]	0.1 [-0.8-0.8]	<0.001
Median HFAZ [IQR]	-0.3 [-1.2-0.6]	-0.6 [-1.6-0.2]	-0.5 [-1.4-0.4]	0.014
Median change in WFAZ between 6 and 12 months [IQR] (*n*=305)	0.1 [-0.5-0.6]	-0.03 [-0.5-0.3]	0.02 [-0.5-0.4]	0.262
Median change in HFAZ between 6 and 12 months [IQR] (*n*=305)	-0.01 [-0.9-0.8]	0.2 [-0.7-0.8]	0.1 [-0.8-0.8]	0.417
Infant feeding categories				
2 months, (n)	344	337	681	
Exclusive breast feeding	143 (42)	163 (48)	306 (45)	
Exclusive formula feeding	81 (24)	11 (3)	92 (14)	<0.001
Mixed feeding	120 (35)	163 (48)	283 (42)	
6 months, (n)	243	274	517	
Exclusive breast feeding	22 (9)	23 (8)	45 (9)	
Exclusive formula feeding	31 (13)	3 (1)	34 (7)	<0.001
Mixed feeding	190 (78)	248 (91)	438 (85)	
12 months, (n)	155	187	342	
Mixed breast feeding	57 (37)	117 (63)	177 (51)	
Mixed formula feeding	91 (59)	53 (28)	149 (43)	<0.001
Solids only	7 (5)	17 (9)	24 (7)	

[a] WFAZ, weight-for-age z-score; HFAZ, height-for-age z-score

Table 3 Prevalence of infant malnutrition

Variable	Mbekweni (Black African)– *n* (%)	TC Newman (Mixed Race) – *n* (%)	Total – *n* (%)	*P*-value
2 months, (n)	344	337	681	
Wasting	22 (6)	9 (3)	31 (5)	0.020
Stunting	42 (12)	73 (22)	115 (17)	0.001
Underweight for age	20 (6)	39 (12)	59 (9)	0.008
Overweight	18 (5)	10 (3)	28 (4)	0.137
Obese	4 (1)	1 (0.3)	5 (0.7)	0.186
6 months, (n)	243	274	517	
Wasting	13 (5)	8 (3)	21 (4)	0.162
Stunting	25 (10)	45 (16)	70 (14)	0.042
Underweight for age	7 (3)	21 (8)	28 (5)	0.016
Overweight	31 (13)	16 (6)	47 (9)	0.006
Obese	17 (7)	8 (3)	25 (5)	0.031
12 months, (n)	155	187	342	
Wasting	3 (2)	9 (5)	12 (4)	0.237
Stunting	12 (8)	31 (17)	43 (13)	0.014
Underweight for age	3 (2)	16 (9)	19 (6)	0.008
Overweight	14 (9)	8 (4)	22 (6)	0.074
Obese	6 (4)	1 (0.5)	7 (2)	0.030

Table 4 Association between maternal factors and infant weight-for-age z-score (WFAZ) at birth

Variable	Median WFAZ [IQR]	Unadjusted regression coefficient [95% CI]	*P*-value	(A) Psychosocial-behavioral model: Adjusted regression coefficient [95% CI]	*P*-value	(B) Nutritional model: Adjusted regression coefficient [95% CI]	*P*-value
Recruitment site							
TC Newman (Mixed race)	-0.7 [-1.4 - -0.1]	Reference		Reference		Reference	
Mbekweni (Black African)	-0.4 [-1.2-0.3]	0.3 [0.2-0.5]	<0.001	0.2 [0.01-0.3]	0.040	0.3 [0.1-0.4]	<0.001
SES quartile							
Highest SES	-0.4 [-1.0- 0.3]	Reference		Reference		Reference	
Moderate-high SES	-0.7 [-1.4- 0.01]	-0.2 [-0.4- -0.01]	0.038	-0.2 [-0.4- -0.03]	0.027	-0.3 [-0.5- -0.1]	0.011
Low-moderate SES	-0.5 [-1.4- 0.04]	-0.2 [-0.4- -0.01]	0.044	-0.2 [-0.4- 0.001]	0.051	-0.2 [-0.5- -0.04]	0.019
Lowest SES	-0.7 [-1.4- 0.04]	-0.2 [-0.4- 0.01]	0.068	-0.2 [-0.4- -0.003]	0.046	-0.3 [-0.5- -0.05]	0.016
Gravidity							
Multigravida	-0.4 [-1.2- 0.2]	Reference					
Primagravida	-0.8 [-1.5- -0.1]	-0.4 [-0.5- -0.2]	<0.001				
Antenatal urine cotinine							
Non-smoker	-0.4 [-1.1- 0.3]	Reference		Reference			
Active smoker	-0.8 [-1.5- -0.2]	-0.4 [-0.6- -0.3]	<0.001	-0.3 [-0.5- -0.1]	0.001		
Antenatal alcohol use							
No self-reported alcohol use	-0.5 [-1.2- 0.2]	Reference		Reference			
Any self-reported alcohol use	-0.9 [-1.6- -0.3]	-0.4 [-0.6- -0.2]	<0.001	-0.3 [-0.5- -0.1]	0.010		
Antenatal depression (EPDS)							
Below threshold	-0.5 [-1.3- 0.1]	Reference					
Above threshold	-0.6 [-1.3- -0.01]	-0.1 [-0.2- 0.1]	0.554				
Recent intimate partner violence							
Below threshold	-0.5 [-1.2- 0.1]	Reference					
Above threshold	-0.6 [-1.4- 0.1]	-0.2 [-0.3- -0.003]	0.045				
Maternal height		0.03 [0.02- 0.04]	<0.001			0.02 [0.01- 0.03]	<0.001

Table 5 Mixed effects model of variables associated with infant weight-for-age z-scores (WFAZ; repeated measurements) at 2 months, 6 months, and 12 months; infants born preterm and/or SGA excluded

Variable	Unadjusted regression coefficient [95% CI]	*P*-value	**Model 1**		**Model 2**	
			Adjusted regression coefficient [95% CI][a]	*P*-value	Adjusted regression coefficient [95% CI][b]	*P*-value
Clinic (*n*=508)						
TC Newman (Mixed Race)	Reference		Reference		Reference	
Mbekweni (Black African)	0.3 [0.2, 0.5]	<0.001	0.4 [0.3, 0.6]	<0.001	0.2 [0.1, 0.4]	0.003
SES quartile (*n*=508)						
Highest SES	Reference		Reference		Reference	
Moderate-high SES	-0.2 [-0.4, 0.1]	0.197	-0.2 [-0.5, 0.01]	0.057	-0.1 [-0.3, 0.2]	0.620
Low-moderate SES	-0.2 [-0.4, 0.1]	0.196	-0.2 [-0.5, -0.01]	0.042	-0.1 [-0.3, 0.1]	0.376
Lowest SES	-0.5 [-0.7, -0.2]	<0.001	-0.6 [-0.8, -0.4]	<0.001	-0.3 [-0.5, -0.1]	0.011
WfA z-score at birth (*n*=508)	0.5 [0.4, 0.6]	<0.001	0.5 [0.4, 0.6]	<0.001	0.5 [0.4, 0.6]	<0.001
Recent intimate partner violence (antenatal; *n*=508)						
Below threshold	Reference		Reference			
Above threshold	-0.1 [-0.3, 0.1]	0.420	-0.02 [-0.2, 0.2]	0.842		
Recent intimate partner violence (early postpartum; *n*=258)						
Below threshold	Reference		Reference			
Above threshold	-0.2 [-0.4, 0.1]	0.297	-0.1 [-0.4, 0.2]	0.382		
Antenatal urine cotinine (*n*=508)						
Non-smoker	Reference		Reference		Reference	
Active smoker	-0.4 [-0.6, -0.2]	<0.001	-0.3 [-0.5, -0.05]	0.015	-0.1 [-0.3, 0.1]	0.312
Early postpartum tobacco use (*n*=258)						
No self-reported tobacco use	Reference		Reference			
Any self-reported tobacco use	-0.3 [-0.6, -0.1]	0.020	0.01 [-0.4, 0.4]	0.969		
Antenatal alcohol use (*n*=508)						
No self-reported alcohol use	Reference		Reference		Reference	
Any self-reported alcohol use	-0.5 [-0.7, -0.2]	<0.001	-0.4 [-0.6, -0.1]	0.001	-0.2 [-0.4, -0.04]	0.019
Early postpartum alcohol use (*n*=258)						
No self-reported alcohol use	Reference		Reference			
Any self-reported alcohol use	-0.1 [-0.5, 0.2]	0.455	0.1 [-0.3, 0.5]	0.657		

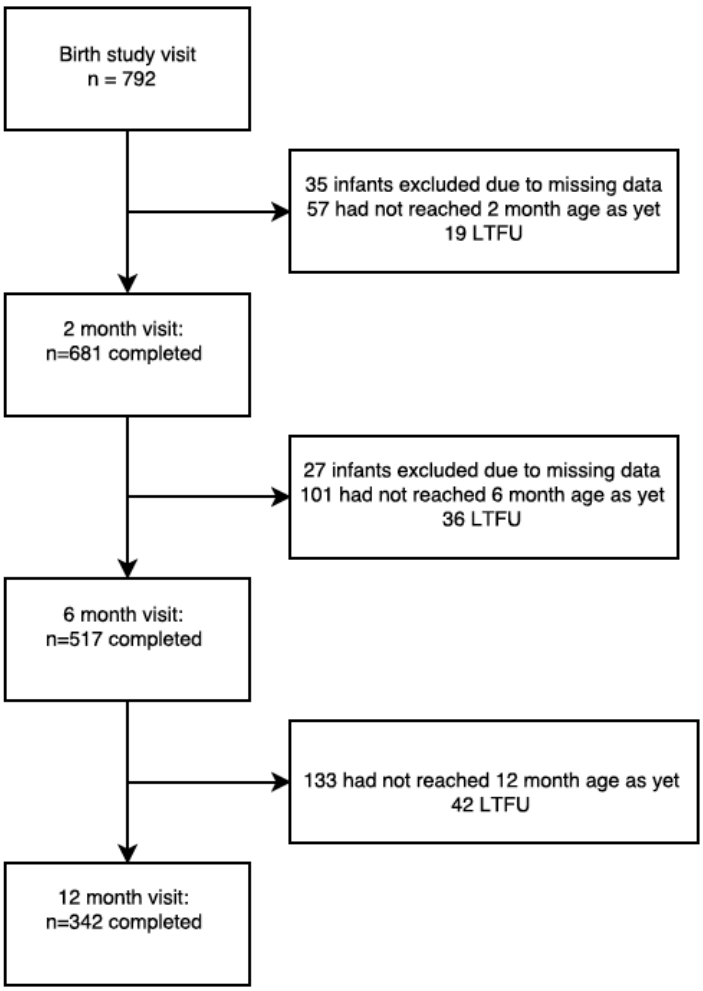
[a] Adjusted for recruitment site and SES quartile; [b] Adjusted for all other covariates in model and for infant age at time of measurement

Table 6 Mixed effects model of variables associated with infant weight-for-age z-scores (WFAZ; repeated measurements) at 2 months, 6 months, and 12 months; restricted to infants born preterm and/or SGA

Variable	Unadjusted regression coefficient [95% CI]	*P*-value	**Model 1**		**Model 2**	
			Adjusted regression coefficient [95% CI][a]	*P*-value	Adjusted regression coefficient [95% CI][b]	*P*-value
Clinic (*n*=196)						
TC Newman (Mixed Race)	Reference		Reference		Reference	
Mbekweni (Black African)	0.8 [0.5, 1.1]	<0.001	0.9 [0.6, 1.3]	<0.001	0.3 [-0.002, 0.7]	0.051
SES quartile (*n*=196)						
Highest SES	Reference		Reference		Reference	
Moderate-high SES	-0.2 [-0.8, 0.4]	0.518	-0.3 [-0.9, 0.2]	0.249	0.01 [-0.4, 0.4]	0.981
Low-moderate SES	-0.3 [-0.8, 0.3]	0.360	-0.6 [-1.1, 0.01]	0.054	-0.1 [-0.6, 0.3]	0.562
Lowest SES	-0.6 [-1.1, 0.004]	0.052	-0.9 [-1.4, -0.3]	0.002	-0.7 [-1.1, -0.3]	0.001
WfA z-score at birth (*n*=196)	0.5 [0.4, 0.6]	<0.001	0.5 [0.3, 0.6]	<0.001	0.5 [0.4, 0.6]	<0.001
Recent intimate partner violence (antenatal; *n*=196)						
Below threshold	Reference		Reference			
Above threshold	-0.4 [-0.8, -0.04]	0.029	-0.1 [-0.5, 0.2]	0.520		
Recent intimate partner violence (early postpartum; *n*=102)						
Below threshold						
Above threshold	Reference		Reference			
	-0.7 [-1.2, -0.2]	0.012	-0.4 [-1.0, 0.2]	0.167		
Antenatal urine cotinine (*n*=196)						
Non-smoker	Reference		Reference		Reference	
Active smoker	-0.8 [-1.2, -0.5]	<0.001	-0.5 [-0.8, -0.1]	0.014	-0.3 [-0.7, -0.03]	0.031
Early postpartum tobacco use (*n*=102)						
No self-reported tobacco use	Reference		Reference			
Any self-reported tobacco use	-0.8 [-1.3, -0.3]	0.002	-0.5 [-1.1, 0.1]	0.093		
Antenatal alcohol use (*n*=196)						
No self-reported alcohol use	Reference		Reference		Reference	
Any self-reported alcohol use	-0.5 [-0.9, -0.1]	0.011	-0.2 [-0.6, 0.2]	0.329	0.1 [-0.3, 0.4]	0.641
Early postpartum alcohol use (*n*=102)						
No self-reported alcohol use	Reference		Reference			
Any self-reported alcohol use	-0.2 [-0.8, 0.4]	0.557	0.1 [-0.5, 0.7]	0.735		

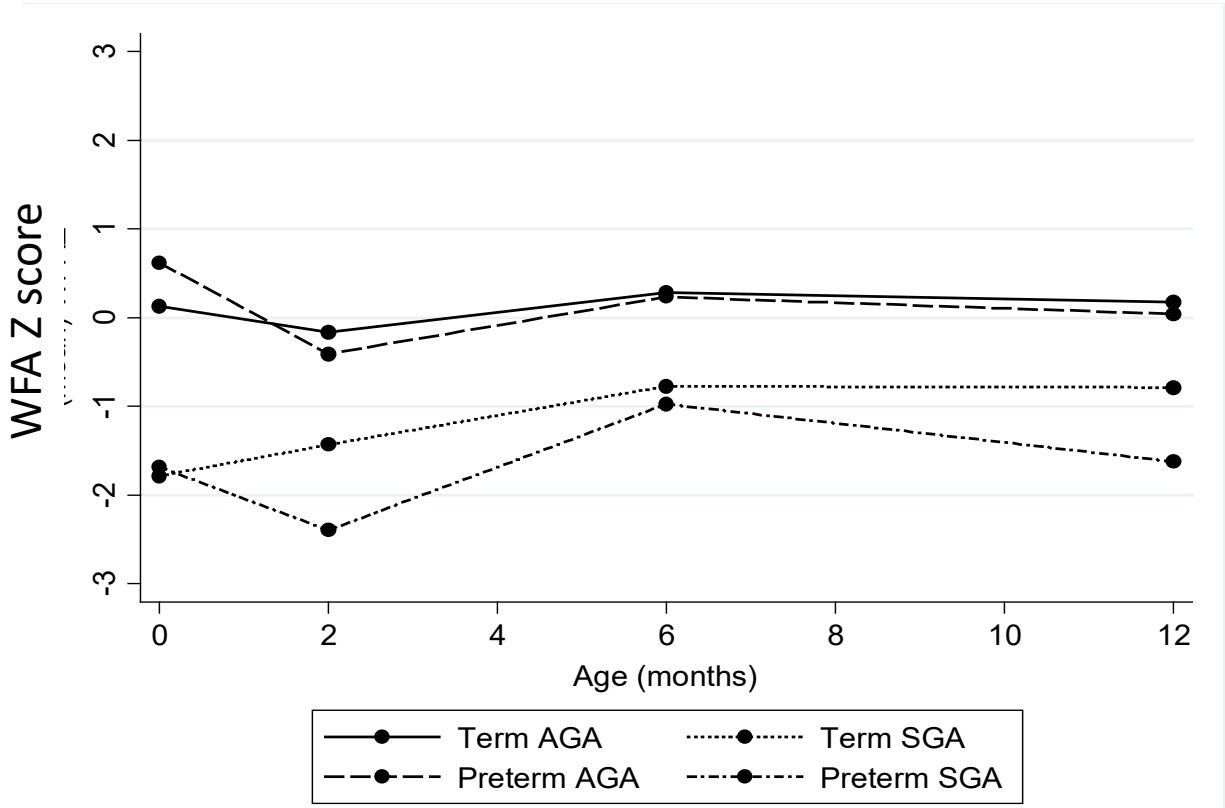
[a] Adjusted for recruitment site and SES quartile; [b] Adjusted for all other covariates in model and for infant age at time of measurement

Figure 1: Flow chart of infants included at each study visit between March 2012 and October 2014



LTFU: loss to follow up

Figure 2: Longitudinal infant WFA z scores stratified by prematurity and weight



Chapter 3: Infant Feeding Practices in a South African Birth Cohort – a longitudinal study

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ABSTRACT

Childhood malnutrition is highly prevalent in low and middle-income countries. The choices of complementary foods, which are important in infant nutrition, are poorly described in this setting. We investigated infant feeding practices in a South African birth cohort, the Drakenstein Child Health Study. Longitudinal feeding data were collected from March 2012 to March 2015. Feeding practices at birth, 6-10 weeks, 14 weeks, 6, 9 and 12 months were investigated using food frequency questionnaires. Anthropometry was measured at birth and 12 months. The quality of the diet was analysed using the WHO infant and young child feeding indicators. Regression models were used to explore associations between feeding and growth outcomes at 1 year. Exclusive breastfeeding (EBF) for 6 months was low (13%) and 19% of infants were introduced to solid foods before 4 months. There was high daily consumption of processed meat (56%) and inappropriate foods such as fruit juice (82%), soft drinks (54%) and refined sugary foods (51%) at 1 year. Dietary diversity and consumption of iron rich foods were low at 6 months (5% and 3% respectively) but higher by 12 months (75% and 78%). Longer duration of EBF was associated with a lower height-for-age z score at 1 year. Several dietary deficits and a rising trend in the consumption of inappropriate, nutritionally poor foods were identified. These findings raise concern about poor dietary practices and the impact on child and long-term health.

KEYWORDS: Birth Cohort; Breastfeeding duration; Complementary feeding; Child feeding; Infant Growth; Low income countries

INTRODUCTION

Childhood malnutrition, encompassing both under- and overnutrition, is highly prevalent in low and middle income countries (LMICs) and is a key contributor to the burden of disease (1). Undernutrition increases the risk of morbidity and mortality with an estimated 45% of child deaths associated with undernutrition in 2011 (1). Inadequate dietary food intake is a key determinant of childhood malnutrition and poor growth.

Recommended infant feeding practices include breast milk feeding from birth to 6 months and introduction of complementary feeds after 6 months (2,3). During this period infants may be particularly susceptible to growth faltering, and deficits acquired at this age are difficult to reverse (2). Furthermore, early nutrition may have a lasting impact on the development of long term health (3,4).

Coupled with high prevalence of under-nutrition, the rates of child obesity in LMICs are likewise rising, termed the double burden of malnutrition (1,5,6). This coincides with global shifts in dietary patterns in LMICs from traditional diets rich in legumes, vegetables and coarse grains to 'Westernised diets' high in refined carbohydrates, sugar, salt and fats (6). These may lead to high levels of consumption of inappropriate foods such as sugar sweetened beverages (SSB) and highly processed foods among infants (7).

South Africa is a society in transition with increasing urbanization and adoption of Western diets. However, few studies have longitudinally investigated patterns of complimentary feeding in this setting. An understanding of such practices is important to inform nutritional determinants of child health and to design interventions to address poor nutrition. The aim of this study was to investigate the feeding practices, the quality of complementary feeding and the association between feeding practices and the nutritional status of infants during the first year of life in a South African birth cohort. We hypothesis that infant feeding practices are suboptimal in this population and that these poor feeding practices are associated with poor infant growth at 1 year.

METHODS

Study design

This study was performed as part of the Drakenstein Child Health Study (DCHS), a birth cohort study in a peri-urban area called Paarl, located 60km outside of Cape Town, South Africa. The primary aim of the DCHS was to investigate the epidemiology, risk factors (including nutrition), aetiology and long-term impact of respiratory illness on child health (8). Detailed longitudinal data on infant feeding practices, complementary feeding and infant anthropometry were collected from birth from March 2012 to March 2015 (8).

Study population

Pregnant women were enrolled during their second trimester from one of two local clinics; TC Newman servicing a predominantly mixed race community and Mbekweni servicing a predominantly Black African community. These are low-income communities with low education levels and seasonal farming employment among women (9). Mother-infant pairs were followed at regular intervals from birth to 12 months (8). Exclusion criteria were woman less than 18 years of age, lack of informed consent or intention to leave the area within 1 year.

Measures

Comprehensive infant feeding data were collected using food frequency questionnaires (FFQ) administered to mothers by trained study staff. Study staff measured infant anthropometry and administered feeding questionnaires at birth and scheduled study visits at 6-10 weeks, 14 weeks, 6 months, 9 months and 12 months.

Feeding data: Detailed longitudinal data were collected on type of milk feed from birth, duration of exclusive breastfeeding (EBF), formula feeding and the introduction and use of complementary foods. Locally validated food frequency questionnaires were used to longitudinally collect complementary feeding data (10). Food lists were piloted and adapted to the local setting with the assistance of community members. The food frequency list for infants from birth till 9 months included 21 individual food items consumed on a daily, weekly and monthly basis. Examples of frequently consumed foods included in questionnaires were cow's milk, yogurt, maize based or commercial porridge. The 12 month food frequency list additionally included starch (potato, rice, pasta, bread, pap), crisps (potato chips), SSB, such as soft drinks and ultra-processed high sugar / high fat foods (sweets / chocolates / pastries). FFQ are the recommended tool when evaluating diets in large sample populations and were utilized in this study (rather than 24-hour dietary recall) (10). Hence, the daily consumption of individual foods recorded in the FFQ was used as a surrogate for a 24-hour dietary recall, which is the recommended tool for the assessment of the WHO Infant and Young Child Feeding (IYCF) indicators (2).

The WHO IYCF indicators were used to assess infant feeding from birth to 12 months of age (2). These include indicators for breastfeeding (EBF under 6 months) and complementary feeding (minimum dietary diversity [MDD], consumption of iron rich foods [IRF]). Although developed to assess the quality of infant diets in population-based studies, these indicators have been utilised by investigators to correlate infant feeding practices with child health outcomes (11,12).

Anthropometry: Infant's weight was measured (to the nearest 10g) in light or no clothing using a Tanita digital platform scale (TAN1584, Illinois, U.S.). Recumbent length was measured using a Seca length-measuring mat (Seca, Hamburg, Germany), performed on a firm surface by two staff members. Mid upper arm circumference was measured at the mid point between the acromion and the olecranon processes of the right arm using a non-stretchable measuring tape. All measurements were performed twice in each child to improve accuracy. Equipment was checked and calibrated weekly. Study staff underwent regular anthropometric training and assessment 3 monthly, conducted by the lead anthropometrist. Inter- and intra-examiner variability was assessed during training session. In

addition, the methodologies for all anthropometric measurements were detailed in the study's standard operating procedure documents.

Socioeconomic status (SES): SES was measured, using a composite score of 4 areas: assets and market access (participants were incrementally scored based on number of specified assets owned and access to retail and financial facilities), household income (incrementally scored based on categorised amounts of combined household incomes per month), employment status (dichotomized as employed or unemployed) and educational attainment (incrementally scored based on level of scholastic achievement), adapted from the South African Stress and Health Study (SASH) (13,14). The composite SES score was determined by summing the scores of each of the above four socio-demographic variables. Participants were stratified into quartiles based on their SES score (low, low-to-moderate, moderate-to-high, or high SES) to compare groups within the study population.

Statistical analysis

Data were analysed using Stata 12 (StataCorp Inc, College Station, Texas, USA).

To explore patterns of complementary feeding, daily consumption of individual foods was expressed using frequency tables. In addition, individual foods were grouped into seven food groups based on the WHO IYCF indicators (grains/roots/tubers; legumes/nuts; dairy; flesh foods; eggs; vitamin A rich foods; other fruits and vegetables) and the frequency of consumption of each food group was calculated (2). Vitamin A rich foods were defined as the consumption of carrots, pumpkin, sweet potatoes or spinach based on the WHO IYCF indicators (2). The frequency of consumption of individual food groups was also explored graphically. MDD was defined as daily consumption of four or more food groups, based on the WHO IYCF indicators (2). The consumption of inappropriate foods was explored by creating an "inappropriate food group" comprising SSB (fruit juices, soft drinks, coffee), crisps, sweets, chocolates, pastries or fried foods based on the WHO Scientific and Technical Advisory Group on Inappropriate Promotion of Foods for Infants and Young Children recommendations (15). Complementary feeding patterns were investigated at three time points: 6, 9 and 12 months. Individual foods, food groups and MDD were explored across SES categories and malnutrition indicators (wasting, stunting and under-weight for age).

Weight for age z score (WFAZ), height for age z score (HFAZ), body mass index (BMI, calculated as weight divided by squared height), BMI for age z score (BMIZ) and mid upper arm circumference (MUAC) for each participant was calculated using the WHO anthro software (16). Linear regression models were used to explore the associations between growth outcomes at 12 months of age (WFAZ; HFAZ; weight for height; BMI for age; MUAC for age) and type of milk feeding (breast vs formula),

duration of EBF, specific food groups and MDD. Logistic regression models were used to explore the associations between infant feeding and overweight/obese at 12 months of age, defined as BMI z-score>2. The selection of exposure variables was based on the literature (11,12,17–19).

Ethics

The Human Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town and the Provincial Child Health Research Committee approved the study. Written informed consent was obtained from mothers.

RESULTS

Study population

Between March 2012 and March 2015, 1,071 mothers gave birth to 1,076 infants. 67 infants (6%) were excluded from this analysis due to incomplete birth anthropometry.

More than half of participants were Black African (55%), table 7. The population was poor with low levels of education; more than 50% of mothers did not complete secondary education. The unemployment rate was high (73%) and average household income low (86% had an average household income less than R5000 / $315 per month). A higher proportion of Black African participants were classified into the lowest and low-moderate SES quartiles compared to mixed race participants (57% vs 43% respectively), table 7. There was a significantly higher HIV prevalence among Black African compared to mixed race mothers (37% vs 3% respectively), table 7.

Within the total study sample, there was a substantial proportion of preterm (17%), low birth weight (16%) or small for gestational age (25%) infants. Most mothers initiated breastfeeding at birth (86%), significantly more among mixed race mothers, table 7. However, only 13% of infants were exclusively breastfed for 6 months or longer. Among mothers who initiated breastfeeding, continued breastfeeding was reported by 60% and 58% at the 9 and 12 month study visits, respectively. Of the 191 HIV-exposed infants for whom feeding data were available, only 87 (46%) were breastfed at birth, compared to 96% of HIV-unexposed infants ($p<0.001$). EBF for 6 months or longer was reported by 26% of the HIV-infected mothers who initiated breastfeeding, compared to 12% of the HIV-uninfected mothers ($p=0.001$). By 6-10 weeks of infant age, 8% of infants were receiving solids; this proportion increased to 19% at 14 weeks of infant age and 73% at 6 months of age.

Overall feeding patterns

Specific feeding patterns were consistently present across the entire study population. The 'grain, root, tubers' group of foods was the most frequent food group fed to most infants at all three time points, figure 3. Daily legumes, nuts or peanut-butter consumption was low at all three time points during infancy. Daily dairy consumption gradually increased from 6 months with most infants (92%) having consumed daily dairy products by 12 months, figure 3.

Daily consumption of IRF or fleshy foods was low at 6 and 9 months but increased by 12 months, figure 3. Most children in the study sample (77%) were consuming some animal protein at 1 year. However, the most common type of fleshy food consumed daily at 12 months was processed meat (56%) followed by red meat (34%). Egg consumption increased significantly between 9 months and 12 months (10% and 61%). Vitamin A rich fruit and vegetables consumption was poor during infancy. Other fruit and vegetable consumption (non-vitamin A rich) increased progressively from 6 months to 1 year with most consuming fruits and vegetables by 1 year.

Inappropriate food consumption was generally high with 13% of infants consuming fruit juices daily at 6 months. By 1 year, almost a third of infants were consuming crisps (32%) and more than half were drinking soft drinks (54%) on a daily basis, table 8. Daily consumption of refined sugary foods (sweets/chocolates/pastries) or fried foods was similarly high at 1 year (51% and 32% respectively). MDD was consistently low at 6 months with only 5% of infants consuming 4 or more food groups daily at this age. Food diversity gradually increased with age to 24% at 9 months and 75% by 12 months.

Comparison of diet among Black African and Mixed-race Infants

Differences in infant feeding practices were observed between the two ethnic groups. Black African infants consumed a higher proportion of maize based cereal at 6 and 9 months compared to mixed race infants, table 8. At 9 months, a significantly higher proportion of Black African children were consuming commercial cereals daily compared to mixed race infants. Consumption of daily dairy products was significantly lower in Black African infants compared to mixed race infants at 6 and 9 months (20% in Black African infants vs. 48% in mixed race infants at 6 months, $p < 0.001$ and 43% Black African vs. 74% mixed race at 9 months, $p < 0.001$). Although daily consumption of fleshy foods was equally low in both groups at 6 and 9 months, by 12 months Black African infants were consuming a higher proportion of animal protein compared to mixed race infants (82% versus 74%; $p=0.034$). Likewise, MDD was higher among Black African infants than mixed race infants at 12 months (80% vs 71%; $p=0.032$). The consumption of inappropriate foods was equally high amongst Black African and mixed race infants at 1 year.

Association between Infant Feeding Practices and Growth

We investigated the associations between growth outcomes at 1 year of age and WHO Infant and Young Child Feeding Indicators (EBF, MDD, IRF) and inappropriate food consumption in unadjusted models, table 9. Only those variables that were significantly associated with growth outcomes in unadjusted analysis were included into the adjusted models. No feeding variables were significantly associated with MUAC and BMIZ and were therefore not analysed in adjusted models. However, longer duration of EBF was associated with a lower WFAZ and HFAZ at 1 year. When adjusted for recruitment site and SES, the association between duration of EBF and WFAZ was not significant (p=0.213) but the association with HFAZ persisted. A one-month increase in the duration of EBF was associated with a 0.1 z-score reduction (95% CI: -0.2, -0.03) in HFAZ at 1 year (p=0.003). MDD and IRF consumption at 6 and 9 months were not associated with growth outcomes at 1 year, table 9. Similarly, inappropriate food consumption at 1 year was not associated with growth outcomes at 1 year. The prevalence of overweight/obesity (BMIZ ≥ 2) at 1 year was 9%. There was no association between daily consumption of fruit juice at 9 months (p=0.916) or inappropriate foods at 12 months (p=0.708) and overweight/obesity at 12 months of age, table 10. Additionally, no association was found between under-consumption of individual food groups or low dietary diversity and indicators of malnutrition such as wasting, stunting and underweight for age (data not shown).

For descriptive purposes, we analysed the frequency of consumption of individual food groups of interest (fleshy foods/IRF and inappropriate foods) and dietary diversity across SES categories, table 11. There were minimal differences in consumption between the highest and lowest socioeconomic groups, table 11. Daily fleshy foods consumption was slightly higher among the highest SES quartile than the lowest (78% vs. 75% at 1 year). Daily fruit juice consumption at 1 year was significantly higher in the highest SES quartile compared to the lowest (91% vs. 74%; p = 0.002). MDD did not differ between the highest and the lowest SES quartiles at any of the three time points, table 11.

DISCUSSION

In this South African birth cohort, we evaluated dietary patterns over the first year of life through comprehensive collection of individual food types, feeding practices and anthropometry. We found low rates of EBF after 3 months, frequent early introduction of complementary foods, low dietary diversity and low consumption of IRF between 6 and 9 months. We observed a concerning pattern of high consumption of inappropriate foods, beginning as early as 6 months. The WHO IYCF indicators (MDD, IRF) were not associated with growth outcomes at 12 months, however a longer duration of

breastfeeding was associated with a lower HFAZ. The consumption of inappropriate foods during infancy was not significantly associated with overweight or obesity at 12 months.

The WHO recommends EBF for 6 months and thereafter continued breastfeeding for up to two years or beyond (20). Despite the proven beneficial effects of EBF on both short and long term health, numerous studies have shown that the prevalence of EBF in LMICs remains low (1,21). Consistent with other South African studies (22,23) we found low rates of EBF, that were even lower compared to other LMICs (18,24,25). The South African guidelines for feeding practices in HIV-exposed infants changed in 2010 from advocating formula based feeding to EBF in mothers on anti-retroviral medication (26). Prior to 2010, free infant formula was provided for HIV exposed infants until 6 months (26). In this study, less than half of HIV exposed infants were started on breast milk at birth, suggesting that implementation of this policy change into clinical practice was slow. Many obstacles to EBF in South Africa remain including maternal perception of safety of breastfeeding in HIV-infected mothers, insufficient breast milk, concern about poor growth, marketing of breast-milk substitutes, maternal work and lack of supportive work structures to promote breast feeding (26,27).

South African studies report 22-80% of infants consume complementary feeds before 6 months (23,28). Consistent with other South African studies (22,28), a high proportion of infants (73%) consumed solid feeds before 6 months. Introduction of complementary feeds earlier than 4 months of age is associated with an increased risk of obesity, infection related morbidity and mortality, associated with allergy and an overall reduction in breastfeeding duration during infancy (3,27). Strategies to promote breastfeeding and counseling of mothers around optimal infant nutrition should be strengthened.

The proportion of infants achieving MDD in our study was substantially lower than previously described (29). Lower SES did not correlate with lower food diversity, but this may reflect the small sample size when stratified by SES and age, table 11. Other factors such as poor maternal nutrition knowledge, delayed introduction of fleshy foods or concerns about potentially allergenic foods may have contributed to these findings (29). Numerous studies have shown the benefits of fleshy foods or IRF on infant growth and cognitive development (4,11,30,31). In this study, processed meats were the most commonly consumed IRF, probably related to lower cost compared to fresh meat. However these are high in sodium and fat; consumption during infancy has been associated with hypertension and coronary artery disease during adulthood (3,4,32,33). There was also frequent consumption of inappropriate foods including crisps and high sugar / high fat foods. Consumption of fruit juices, cool drinks and ultra-processed sweet snacks may increase the risks of growth faltering, obesity and type 2 diabetes during childhood and has been shown to increase the infant's preference for sugary foods

and drinks which persist into childhood and adolescence (3,7,34–35). In the context of rising obesity rates and non-communicable diseases in LMICs, these findings have important implications for dietary education (36).

We found no association between patterns of complimentary feeding and growth outcomes at 1 year except that duration of EBF was significantly associated with a lower HFAZ at 1 year. Similar findings have been reported and may reflect a transient slowing in length attainment at 12 months (18,37,38). Further evaluation of infant length at subsequent age points in exclusively breastfed infants is required.

Limitations of this study include the lack of feeding information in food frequency questionnaires that prevented the calculation of some WHO IYCF indicators including minimum meal frequency and minimum adequate diet. This may partly explain our finding of the lack of association between MDD, consumption of IRF and growth outcomes during infancy. Other studies have highlighted the limitations of the WHO IYCF indicators in the analysis of causal pathways to child growth (12,18). However, our study included comprehensive repeated measures on feeding. These findings may not reflect the infant feeding patterns in other LMICs as dietary practices vary by culture and location. Nevertheless, it is likely that these feeding patterns may occur in other African settings, given the changes in lifestyle and urbanisation. Finally, we only assessed outcomes until 1 year of age; long-term follow-up of children is being undertaken to investigate the impact of early childhood nutrition on child health.

We demonstrated deficits in feeding practices and poor quality of the complementary foods in peri-urban South African infants. Utilising the WHO IYCF indicators, we found low dietary diversity and low consumption of IRF foods between 6 and 9 months of age. However, these indicators improved by 12 month of age and were not associated with most growth outcomes at 12 months. Our finding of the low rates of EBF in this study population, the early introduction of complementary foods and frequent, early consumption of inappropriate complementary foods raises concerns about the impact of such dietary practices on child and subsequent adult health. Educational initiatives to promote a healthy diet should be strengthened, targeting pregnant women and mothers.

KEY MESSAGES:

1. Based on the WHO IYCF indicators, the quality of infants' diet in this poor peri-urban population appears suboptimal, however, these indicators did not correlate with growth outcomes at 12 months.

2. The low prevalence of EBF during the first 6 months of life is concerning particularly in a high HIV prevalence setting and suggests the need for strengthening of programs and strategies to promote EBF.
3. There is a concerning high prevalence of consumption of poorly nutritious foods such as processed meats, crisps and SSB in infancy.

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Table 8 Daily consumption of WHO food groups and individual food items, stratified by site in infants receiving solids; results presented as *n* (%) or *n* [%; 95% CI] where proportions consuming food groups and median food groups consumed are compared across site

	6 months		9 months		12 months	
Variable	Mbekweni (*n*=221)	TC Newman (*n*=290)	Mbekweni (*n*=261)	TC Newman (*n*=304)	Mbekweni (*n*=204)	TC Newman (*n*=257)
Grains, roots and tubers	212 [96; 92, 98]	265 [91; 88, 94]*	244 [93; 90, 96]	246 [81; 76, 85]**	195 [96; 92, 98]	247 [96; 93, 98]
Porridge (maize)	44 (20)	27 (9)	116 (44)	72 (24)	-	-
Baby cereal (commercial)	203 (92)	238 (82)	212 (81)	171 (56)	-	-
Starch (potatoes, rice, pasta, bread, mielie-pap)		-	-	-	195 (96)	247 (96)
Legumes and nuts	2 [0.9; 0.1, 3]	6 [2; 0.8, 4]	23 [9; 6, 13]	45 [15; 11, 19]*	12 [6; 3, 10]	6 [2; 0.9, 5]
Peanut butter	2 (0.9)	2 (0.7)	17 (7)	40 (13)	-	-
Nuts	0 (0)	1 (0.3)	1 (0.4)	0 (0)	-	-
Dairy products (cow's milk/powder milk/yogurt/maas)	44 [20; 15, 26]	140 [48; 42, 54]**	113 [43; 37, 50]	225 [74; 69, 79]**	189 [93; 88, 96]	236 [92; 88, 95]
Flesh foods	6 [3; 1, 6]	11 [4; 2, 7]	57 [22; 17, 27]	58 [19; 15, 24]	167 [82; 76, 87]	189 [74; 68, 79]*
Chicken	3 (1)	5 (2)	45 (17)	32 (11)	68 (33)	57 (22)
Red meat	1 (0.5)	0 (0)	1 (0.4)	3 (1)	71 (35)	87 (34)
Organ meat	0 (0)	0 (0)	6 (2)	5 (2)	9 (4)	7 (3)
Fish	1 (0.5)	2 (0.7)	6 (2)	14 (5)	8 (4)	10 (4)
Processed meat	2 (0.9)	8 (3)	21 (8)	31 (10)	120 (59)	140 (54)
Eggs	3 [1; 0.3, 4]	11 [4; 2, 7]	20 [8; 5, 12]	36 [12; 8, 16]	139 [68; 61, 74]	143 [56; 49, 62]*
Vitamin-A rich fruits and vegetables	10 [5; 2, 8]	50 [17; 13, 22]**	42 [16; 12, 21]	87 [29; 24, 34]**	-	-
Other fruits and vegetables	102 [46; 39, 53]	52 [18; 14, 23]**	153 [59; 52, 65]	116 [38; 33, 44]**	190 [93; 89, 96]	236 [92; 88, 95]
Inappropriate foods	33 [15; 11, 20]	31 [11; 7, 15]	73 [28; 23, 34]	93 [31; 25, 36]	185 [91; 86, 94]	235 [91; 87, 95]
Fruit juice (also included in Other fruits and vegetables)	33 (15)	31 (11)	73 (28)	93 (31)	173 (85)	207 (81)
Crisps	-	-	-	-	62 (30)	87 (34)
Coffee	-	-	-	-	22 (11)	22 (9)

Cold drinks	-	-	-	-	110 (54)	140 (54)
Sweets / chocolates/ pastries	-	-	-	-	90 (44)	143 (56)
Fried foods	-	-	-	-	71 (35)	75 (29)
Food diversity						
Median food groups consumed daily [IQR]	2 [1, 2]	2 [1, 2]	2 [2, 3]	3 [2, 4]	5 [4, 5]	4 [3, 5]*
≥4 food groups consumed daily	8 (4)	16 (6)	56 (21)	80 (26)	163 (80)	183 (71)

(-) indicates that consumption of food item is not assessed at time point; Mbekweni=Black/African community, TC Newman=Mixed race community; * $p<0.05$; **$p<0.001$

Table 9 Linear regression models exploring variables associated with infant growth outcomes at 12 months of age (*n*=453); results presented as unadjusted and adjusted regression coefficients [95% CI]

Variable	MUACZ[2] (Unadjusted)	BMIZ[3] (Unadjusted)	HFAZ[4]		WFAZ[5]	
			Unadjusted	Adjusted[6]	Unadjusted	Adjusted[6]
Months of EBF (*n*=386)[1]	-0.04 [-0.1, 0.01]	-0.001 [-0.1, 0.1]	-0.1 [-0.2, -0.1]**	-0.1 [-0.2, -0.03]*	-0.1 [-0.1, -0.01]*	-0.1 [-0.1, 0.01]
Food diversity score at 9 months (*n*=398)	0.02 [-0.1, 0.1]	0.04 [-0.05, 0.1]	0.04 [-0.05, 0.1]		0.1 [-0.04, 0.1]	
Food diversity score at 12 months	0.1 [-0.04, 0.2]	0.1 [-0.1, 0.2]	0.1 [-0.1, 0.2]		0.1 [-0.04, 0.2]	
Animal source protein at 9 months (*n*=398)						
Consumed less than daily	Reference	Reference	Reference		Reference	
Consumed daily	0.1 [-0.2, 0.3]	0.1 [-0.2, 0.4]	0.04 [-0.3, 0.3]		0.1 [-0.2, 0.4]	
Inappropriate foods consumed at 12 months						
Consumed less than daily	Reference	Reference	Reference		Reference	
Consumed daily	-0.1 [-0.5, 0.3]	-0.01 [-0.4, 0.4]	0.2 [-0.3, 0.6]		0.1 [-0.3, 0.5]	

[1] Restricted to mothers who initiated breastfeeding and who reported cessation of EBF during the study period; * p<0.05; **p<0.001

[2]Mid-upper-arm circumference; [3]Body-mass-index-for-age z score; [4]Height-for-age z score; [5]Weight-for-age z score

[6]Adjusted for recruitment site, SES and duration of EBF

Table 10 Variables associated with overweight/obese at 12 months of age (*n*=453)

Variable	Normal BMIZ[2] – *n* (%; *n*=412)	Overweight/obese – *n* (%; *n*=41)	Unadjusted odds ratio [95% CI]	*p*-value	Adjusted[4] odds ratio [95% CI]	*p*-value
Recruitment site						
Mbekweni	171 (86)	29 (15)	Reference		Reference	
TC Newman	241 (95)	12 (5)	0.3 [0.1, 0.6]	0.001	0.2 [0.1, 0.5]	<0.001
SES quartile						
Highest SES	75 (89)	9 (11)	Reference		Reference	
Moderate-high SES	105 (85)	18 (15)	1.4 [0.6, 3.4]	0.413	1.3 [0.5, 3.2]	0.581
Low-moderate SES	116 (93)	9 (7)	0.6 [0.2, 1.7]	0.337	0.4 [0.2, 1.2]	0.117
Lowest SES	116 (96)	5 (4)	0.4 [0.1, 1.1]	0.076	0.2 [0.1, 0.7]	0.010
Months of EBF (*n*=386)[1]			1.1 [0.9, 1.3]	0.452		
Fruit juice consumed at 9 months (*n*=398)						
Consumed less than daily	243 (91)	23 (9)	Reference			
Consumed daily	121 (92)	11 (8)	1.0 [0.5, 2.0]	0.916		
Poor quality foods consumed at 12 months						
Consumed less than daily	34 (89)	4 (11)	Reference			
Consumed daily	377 (91)	36 (9)	0.8 [0.3, 2.4]	0.708		
WFAZ[3] at birth			1.3 [1.0, 1.8]	0.027	1.3 [1.0, 1.7]	0.075

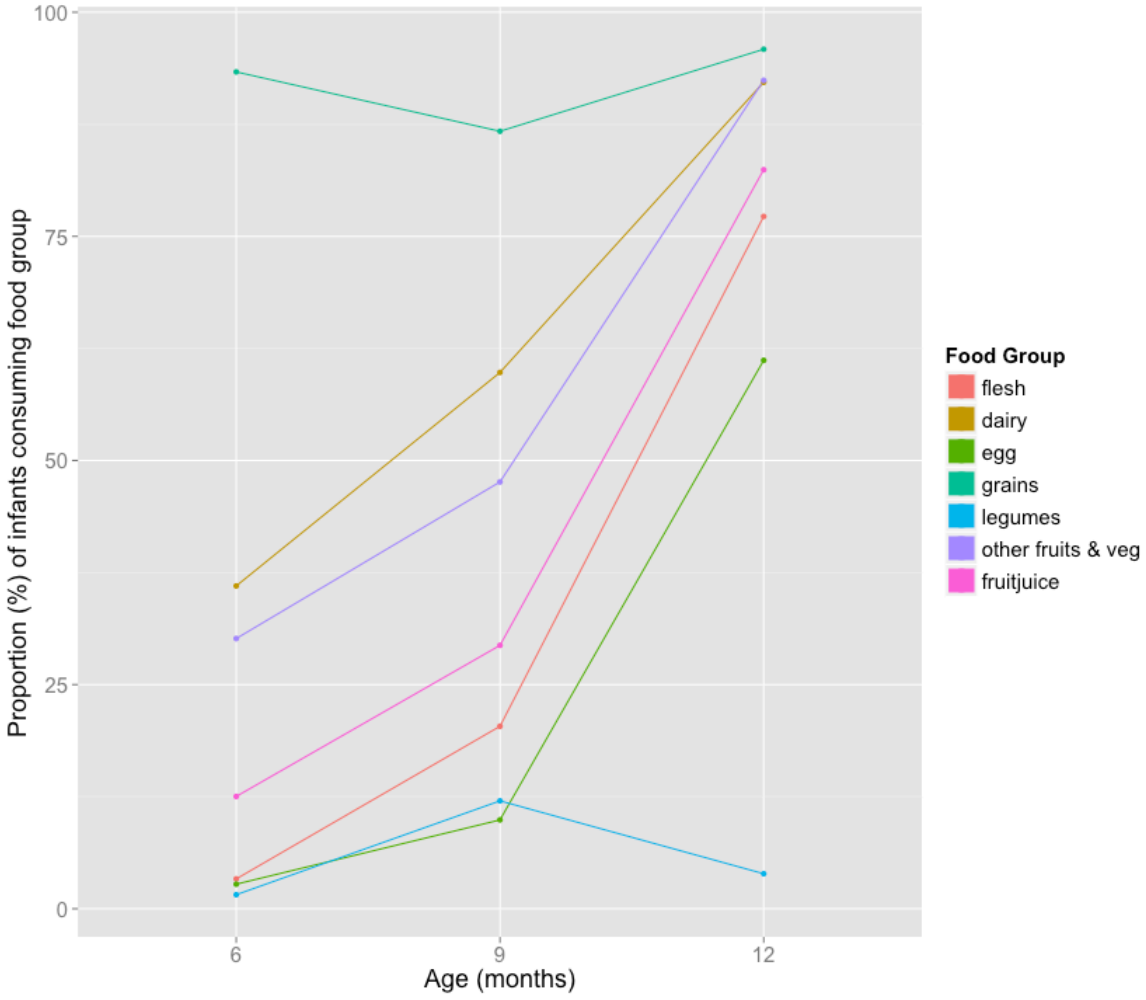
[1]Restricted to mothers who initiated breastfeeding and who reported cessation of EBF during the study period; [2]Body-mass-index-for-age z score; [3]Weight-for-age z score; [4]Adjusted for recruitment site, SES and WFAZ at birth.

Table 11 Individual foods and food groups stratified by Socioeconomic Status (SES), results presented as *n* (%) or *n* [%; 95% CI] where proportions and median food groups consumed are compared across SES categories

	6 months		9 months		12 months	
Variables	Lowest SES (*n*=143)	Highest SES (*n*=107)	Lowest SES (*n*=154)	Highest SES (*n*=113)	Lowest SES (*n*=125)	Highest SES (*n*=87)
Flesh foods	6 [4; 2, 9]	3 [3; 0.6, 8]	27 [18; 12, 24]	19 [17; 10, 25]	94 [75; 67, 82]	68 [78; 68, 86]
Inappropriate foods						
Fruit Juice	25 [17; 12, 25]	8 [7; 3, 14]*	36 [23; 17, 31]	33 [29; 21, 39]	92 [74; 65, 81]	79 [91; 83, 96]*
Food diversity						
Food group score – median (IQR)	2 (1 – 2)	2 (1 – 2)	2 (1 – 3)	2 (2 – 3)	5 (3 – 5)	5 (3 – 5)
Food diversity						
≥4 food groups	9 [6; 3, 12]	6 [6; 2, 12]	36 [23; 17, 31]	25 [22; 15, 31]	90 [72; 63, 80]	65 [75; 64, 83]

(-) indicates that consumption of food item/food group is not assessed at time point

Figure 3: Daily consumption of food groups during infancy



Chapter 4: The Association of the Intestinal Microbiome with Childhood Stunting: Findings from a South African Birth Cohort

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Abstract

Background

Stunting contributes a significant burden to child health globally, disproportionately affecting children in low- and middle-income countries despite preventative nutritional and environmental interventions. Evidence suggests that the intestinal microbiome contributes to the pathogenesis of stunting, however, few studies have characterized the microbiome longitudinally in stunted African children. This study describes the early gut microbiome of South African children with stunting compared to those with normal linear growth.

Methods

Children in the Drakenstein Child Health Study, a birth cohort in a low income, peri-urban setting, had stool collected during the first 3 years of life. Anthropometry was measured during routine study visits. Intestinal microbiome was characterized using 16S rRNA gene amplicon sequencing of longitudinally collected stool samples. A linear mixed effects model was used for assessment of alpha diversity and PERMANOVA for beta diversity. Significant differences in microbial relative abundance between the 2 groups were assessed using MaAsLin, accounting for repeat stool sampling and potential microbiome confounders.

Results

A subset of 138 children (45% female) with complete clinical and microbial data was analyzed. Most participants (70%) had more than 1 stool sample collected at different time points, with the mean age of stool collection being 8.7 months (range 0-40). Stunting (height-for-age z score < -2) was found in 34 (25%) children of which 19 (14%) had severe (HAZ < -3). A total of 401 stool samples were analyzed. There were significant differences in alpha (p=0.009) and beta diversity (p=0.001) between severely stunted and normal height groups, after adjusting for method of stool collection and age. At phylum level, children with stunting had greater abundance of *Proteobacteria* in the first 4 months of life and were depleted in *Bacteroidetes.* On MaAsLin analysis, the genus *Ruminococcus* was significantly reduced in the stunted group (q=0.03), specifically, *Ruminococcus gnavus* on species level analysis (q=0.13).

Conclusion

We found a distinct microbial signature associated with stunting. Specifically, Ruminococcus *gnavus*, an organism previously shown to be a butyrate producer and a growth discriminatory microbe in animal studies of undernutrition, was significantly depleted in the stunted group. These findings could inform the development of future microbial therapeutics in children with stunting.

Keywords: Malnutrition, stunting, microbiome, low-middle income, African

BACKGROUND

Malnutrition is a leading contributor to mortality in children, with approximately half of deaths in children under 5 years being attributable to undernutrition. Stunting, defined as a WHO length/height-for-age z-score of less than -2, remains a considerable challenge to healthcare systems in LMIC countries (1,2). In 2018, approximately 49 million children were wasted (defined as a WHO weight-for-height z-score of less than -2) and 149 million had stunting globally (1). Stunting leads to significant negative impacts on health including increased risk of mortality, reduced cognitive function and a higher association with the development of chronic disease during adult life (3). Although global rates of stunting appear to be decreasing, the rates of stunting in Africa continues to rise with recent global events expected to worsen rates of childhood malnutrition (4). In South Africa, the prevalence of stunting remains high, affecting approximately 22% of children under 5 years (5).

Global strategies aimed at reducing malnutrition have had limited impact. Nutritional supplementation is often insufficient to correct the linear growth deficit in children with stunting, with only 10% response in linear growth deficit. In a large severe acute malnutrition study investigators reported that up to 30% of children failed standard nutritional rehabilitation (6). Furthermore, several interventions aimed at treating stunting, including maternal/pregnancy interventions, dietary modifications and micronutrient supplementation have resulted in minimal or no improvement in growth outcomes (3,7). Larger scale, community directed interventions such as the WASH study aimed at improving drinking water, hygiene and sanitation and the SHINE trial which included multiple interventional arms comprising WASH interventions, ready-to-use-therapeutic feeds (RUTF), complementary feeding and various combinations of these interventions, surprisingly demonstrated no effect on linear growth outcomes (8). The failure of these large-scale interventions

emphasizes the complexity of stunting pathogenesis and suggests that more novel therapeutics need to be explored.

The etiology of malnutrition, specifically stunting is multifactorial including a complex interplay between chronic energy or food deprivation, enteric infections, environmental microbial burden, host inflammation and immune dysfunction (3,9). There is growing evidence that the gut microbiome contributes to the development and persistence of malnutrition (10–12). Smith et. al. described a causal role of the microbiome in the development of kwashiorkor in Malawian twins (13). Studies have shown disruption of the microbial community (dysbiosis) and impaired maturation of the microbiome in malnourished children (stunted and wasted) which persists despite the introduction of ready-to-use-therapeutic-feeds (11). Animal studies have shown that specific consortia of bacteria can ameliorate the malnourished phenotype (12). A recent study using microbiota-directed complementary food to manipulate the gut microbiome in children with moderate acute malnutrition showed a more rapid improvement in weight-for length and weight-for-age compared to children receiving ready-to-use supplementary feeds (14). This was the first study to demonstrate the efficacy of a microbiome directed approach to treating malnutrition.

There are a limited number of studies that have explored the microbiome in children with stunting. Several studies (15–18) reveal an emerging microbiome pattern in stunted children; however, they are limited by their small sample sizes, cross-sectional nature of study design or limited microbiome analysis, with several studies not adjusting for important microbiome modifying confounders such as age. A recent large study in rural Zimbabwean children showed that functional metabolic features were moderately predictive of attained linear growth, further supporting the vital role of the microbiome in the pathogenesis of stunting (19).

The gut microbiome has been shown to vary significantly by geographic location, likely driven by variation in diet, healthcare practices (e.g., antibiotic exposure), and environmental exposures (urban versus rural populations) (20,21). To date, only 2 studies have investigated the gut microbiome of children with stunting and none with wasting in sub-Saharan Africa, with no studies from South Africa, where the burden of disease remains high. Further characterization of the gut microbiome in children residing in different African geographies is required. This study aims to explore the microbiome in longitudinally sampled children with stunting compared to those with normal growth, in a South African birth cohort.

METHODS

Study population

We analyzed a subgroup of participants enrolled on the Drakenstein Child Health Study (DCHS), a birth cohort investigating the early life determinants of child health in a peri-urban area 60 kilometres from Cape Town, South Africa. Detailed study design has been published elsewhere (22). Briefly, pregnant women were recruited at their antenatal visit, followed through pregnancy and mother-child dyads were followed from birth through childhood. Mothers were eligible for enrollment if they were older than 18 years, between 20-28 weeks gestational age at enrolment and were planning to remain in the Drakenstein area. The study population was predominantly from 2 low-income areas in close proximity, Mbekweni and TC Newman.

Study design

This descriptive study included a subset of children on the DCHS (most with longitudinal stool samples). Children that had one or more WHO defined pneumonia episodes between birth and 3 years of age were identified for analysis based on the availability and method of stool sample collection preceding, at diagnosis, and following their pneumonia episode. Non-pneumonia controls were a subset of children on the DCHS between birth and 2 years of age with no reported pneumonia or wheezing episodes, who had existing gut microbiome 16S rRNA gene amplicon sequencing performed at routine study visits as part of a separate microbiome sub-study.

Study staff collected clinical data, stool samples and measured anthropometry at frequent study visits during the first year of life (6 weeks, 6 months, 12 months) according to standard procedures. Thereafter, study visits and specimen collections occurred 6 monthly, Figure 4.

Measures

Birth weight and length was abstracted from infants' hospital folders after training labor-ward staff in anthropometric measurements. Gestational age at birth was determined using 2nd trimester ultrasound and where this was not available, last menstrual period was used.

Anthropometric measurements included weight, height/length, mid-upper arm circumference, skin fold thickness and head circumference. Measurements were conducted by trained study staff based on standard procedures. Study staff underwent regular anthropometric measurement training during which staff measurements were compared to those from the lead anthropometrist. Weight was

measured (to the nearest 0.1kg) using a Tanita Scale (Tokyo, Japan) and height (to the nearest 0.5cm) using a SECA measuring mat in children under 2 years and stadiometer (Hamburg, Deutschland) in those older than 2 years. All children were measured twice and if the reading were discordant with each other, a third measurement was performed and documented as the final measurement.

During pneumonia episodes, stool samples were collected at the time of diagnosis (ideally prior to antibiotic administration) and 4-6 weeks following the pneumonia episodes. A stool sample preceding the pneumonia episode was identified by using the sample collected at the most recent routine study visit preceding the pneumonia episode. Participants had their stool samples collected by study staff at routine study visits and during pneumonia episodes. Stool samples were either collected from the child's diaper using sterile spatula and a screw-top container or stool was aspirated from the rectum using a sterile rectal tube. The method of stool collection was documented for each sample. The stool specimens were initially stored at -20℃ onsite for up to 6 hours. Specimens were then transported in a temperature control cooler to the laboratory where they were placed in a -80˚C freezer until further processing.

Clinical variables that are known to be associated with microbial perturbation during infancy were collected using case report forms administered by study staff at visits and included, age at stool collection, sex, mode of delivery (vaginal versus caesarian section), infant birth outcomes (prematurity, birth weight), infant milk feeding modality (exclusive breast, exclusive formula or mixed feeding), antibiotic exposure and infectious episodes. Antibiotic exposure was defined as any antibiotic exposure during the first year of life. Socio-economic status (SES) was determined using a validated tool which calculated a composite score based on 4 variables associated with SES (household income, maternal education, asset ownership, household size) SES was stratified into quartiles to facilitate comparison within the study population (23).

DNA extraction and sequencing

Sequencing procedures on the DCHS were previously described elsewhere (24). Briefly, stool specimens were thawed, and DNA extraction was done using QIAsymphony DSP Virus/Pathogen Mini Kit® (Qiagen GmbH, Hilden, Germany) as previously described. Thereafter, specimens underwent PCR amplification of the V4 hypervariable region of the 16S rRNA bacterial gene and library preparation was performed as previously described. The 16S library from faecal specimens was sequenced using the MiSeq Reagent Kit v3, 600 cycles (Illumina, CA, USA). The denatured library was loaded to the Illumina® MiSeqTM platform as per manufacturer's instructions.

Bioinformatics analysis

Fastq files generated from the Illumina platform were further processed using QIIME2. Sequences were demultiplexed using the 'dmux' function and thereafter DADA2 was used to quality filter, trimmed and denoise sequences to generate amplicon sequence variants (ASV) (25). Taxonomic identification was performed using the reference Greengenes database in QIIME2 (26). The ASV feature-table and taxonomic data were further analyzed using Phyloseq package (Version 4.2.2), in R (Version 4.2.2).

STATISTICAL ANALYSIS

Clinical metadata

Continuous data were reported as means and standard deviations or medians and range depending on distribution of data. Categorical data were reported as proportions. Student t-test was used for statistical comparison of continuous data and Chi squared test for comparison of categorical data.

Definition of stunting: Given the longitudinal nature of sample collection in this study and the dynamic nature of stunting (a participant may have an initial normal height then becomes stunted at subsequent timepoints or vice versa), we defined the stunted group as any stool sample that was associated with a WHZ <-2. The normal height group was defined as any stool sample that was associated with a WHZ >-2. In addition, participants were classified as 'ever stunted' if they had a WHZ < -2 at any point in time. To provide a further insight into the prevalence of stunting at a single timepoint in this study population, the proportion of children with stunting (WHZ <-2) at 11 months was also determined (timepoint with the highest density of anthropometry measures), using cross-sectional height measurements at 11 months (+- 1 month).

Microbiome statistical analysis

Shannon index was used to assess alpha diversity in Phyloseq. Shannon diversity was assessed visually across all continuous and categorical covariates. Significant differences in alpha diversity within covariates of interest (stunting, under-weight-for-age, wasting) were assessed using a mixed-effects linear model, which enabled adjustment for the longitudinal nature of sample collection and adjustment for potential microbiome confounders such as age, sex and method of stool collection.

Beta diversity was assessed using unweighted Unifrac distances between categories of covariates of interest and were represented graphically on a principle coordinates plot. PERMANOVA (999

permutations) was used to determine statistical significance of beta diversity between groups while adjusting for confounders.

Compositional differences in the microbial community between categories of covariates of interest were determined by calculating the relative abundance of OTUs at different phylogenetic levels (phylum, genus) which were then represented as proportions or graphically as stacked stream plots in R using the packages Phyloseq and ggplot.

Significant differences in microbial relative abundance among clinical covariates of interest was determined using the statistical pipeline, MaAsLin which performs boosted additive general linear modeling per OTU (27). The pipeline supports adjustment for potential confounders such as age, and method of sample collection together with mixed-effects modeling which enables adjustment for repeated sampling.

RESULTS

We analyzed a subset of 138 children with complete clinical and microbial data, who had stool samples collected between 11 September 2012 to 26 April 2016, table 12. The majority of participants (70%) had more than one stool sample collected at different time points.

The mean age at stool collection was 8.5 months (SD =6.4, range 1 - 40 months), with most participants (87%) under 12 months at time of stool collection. Most participants were male (55%) and 65% lived in low to low-moderate SES. A substantial proportion of infants (29%) were HIV exposed, however, none were HIV infected.

Normal vaginal (vertex) delivery (NVD) was the dominant method of delivery (82%) with obstetric emergencies being the main indication for performing a caesarian section (56%). Most infants were born at term (76%), with a mean birth weight of 2900 grams. Almost a quarter of participants (24%) had received antibiotics at the time of stool collection, the most frequent being Amoxicillin or Ampicillin (79%). Almost three-quarters (74%) of participants were ever breast-fed but only 17% received exclusive breastfeeding for at least 5 months.

More than a third of the study population (35%) were classified as stunted at any one point in time (ever stunted), with 20% of these stunted participants being classified as severely stunted (WHZ < -3)

at one or more time points, table 13. At the 10-month cross-sectional timepoint, 25% of participants were stunted, with more being classified as severely stunted versus moderately stunted (14% versus 11%). Only 15% of the study population was found to be ever under-weight-for-age and 9% were ever wasted.

There were 104 stool samples that were analyzed in the stunted group and 278 samples in the non-stunted group, table 13 and supplementary table 14. Comparison of the clinical data associated with stool samples from the stunted and non-stunted groups showed that the groups were mostly similar, supplementary table 14. There was an equal distribution of stool collection methods ($p = 0.13$), sex ($p = 0.25$), SES ($p = 0.07$), history of antibiotic use ($p = 0.94$) and breastmilk exposure (0.62) between the two groups. There were however differences in the distribution of delivery modality with a higher proportion of cesarean sections in the stunted group ($p = <0.05$), a higher proportion of HIV exposed infants in the non-stunted group ($p = <0.05$) and a larger proportion of preterm births in the stunted group ($p = <0.05$).

Microbiome analysis

A total of 1366 OTUs from 401 samples were analyzed after quality filtering. There were approximately 15.5 million reads in total, with a range between 5090 and 170193 and a mean of 39 000 reads per sample.

Evaluation of alpha diversity in variables of interest:

Alpha diversity, measured using the Shannon diversity index, was analyzed across variables known to be associated with the gut microbiome such as age, sex, mode of delivery, breast milk exposure, SES, prematurity and antibiotic exposure. Only age was found to be significantly associated with alpha diversity ($p < 0.005$) on mixed effects linear modeling, after adjusting for method of stool collection. Participant sex ($p = 0.14$), mode of delivery ($p = 0.17$), breastmilk exposure ($p = 0.16$), antibiotic exposure ($p = 0.64$), SES and prematurity were not significantly associated with alpha diversity after adjusting for age and method of stool collection.

Shannon alpha diversity was significantly lower in the severely stunted group compared with the normal height group ($p = 0.009$) after adjusting for repeated samples, age, sex, mode of delivery, method of stool collection, SES, breastmilk exposure and history of antibiotic use, figure 5A. In addition, alpha diversity was positively associated with height-for-age z score ($p = 0.002$), after adjusting for repeated samples, age and method of stool collection.

Differences in Beta diversity between stunted and controls:

Beta diversity analysis, measuring unweighted Unifrac distance, showed a significant difference between severely stunted and normal height children (PERMANOVA, p = 0.001) after adjusting for age, mode of delivery, method of stool collection, breastmilk exposure and antibiotic use, figure 6.

Community composition in stunted versus normal height controls:

Analysis of community composition at phylum level showed that *Actinobacter* was dominant at phylum level in the study population, especially during the first 6 months of life, during which children were exposed to breastmilk, figure 7A. *Proteobacteria* was dominant early on (first 5 months), thereafter abundance levels decreased progressively. Firmicutes were the third most abundant during the first 5 months, but progressively rose thereafter, to become the most dominant phylum after 6 months. *Bacteroidetes* was under-represented in comparison to the other 3 phyla during the first year, with abundance gradually increasing with age. In stunted children, *Actinobacter* was also dominant at phylum level. Compared to normal height children, the stunted group appeared to have a higher *Proteobacteria* abundance in the first 3 months, which then declines to similar levels as the normal height group.

At genus level *Ruminococcus* abundance was consistently lower in the stunted group over the first 2 years (figure 12, supplementary material) and was significantly depleted in the stunted group compared to the normal height group (MaAsLin, q= 0.056) after adjusting for repeated samples, age, method of stool collection, SES, breastfeeding and history of antibiotic use, figure 8. Furthermore, species level analysis with MaAsLin showed that *Ruminococcus gnavus,* was significantly depleted in the stunted group compared to the normal height group (q = 0.13) after adjusting for repeated samples, age and method of stool collection. When stunting was stratified by severity (normal height, moderate and severe), the genus *Clostridium* was significantly depleted in the severely stunted group compared to the moderately stunted group (q = 0.07).

DISCUSSION

In this novel study of the microbiome in stunted South African children residing in a low socio-economic setting, we found significant differences in the microbiome between stunted compared to children with normal linear growth. Severe stunting was associated with a significantly lower alpha diversity compared to the normal height group and higher HAZ was positively associated with higher

alpha diversity. Overall bacterial community composition in samples from children with severe stunting was significantly different compared to those of normal height on beta diversity analysis. Taxanomic composition was mostly similar between the groups at phylum level. However, at genus level, *Ruminococcus,* and, at species level, *Ruminococcus gnavus,* were significantly depleted in the stunted group compared to the normal height group. In addition, the genus *Clostridium* was significantly depleted in the severely stunted group compared to the moderately stunted group.

The findings of lower alpha diversity in the stunted group compared to healthy controls is consistent with a previous study of the microbiome in a small cohort of healthy and stunted Malawian and Bangladeshi children (28). In contrast, several other studies evaluating the microbiome in stunted children showed no difference in alpha diversity between stunted and non-stunted groups (15,16,18). These studies may have been limited by small sample sizes and cross-sectional sampling and may have been underpowered to detect a difference between groups. Furthermore, the study populations differed by age, risk factors and geography, all relevant exposures that may impact alpha diversity. The study by Dinh comprised a highly selective population of low-birth-weight infants with persistent stunting (15). Vonaesch et al. included a large study population with cross-sectional stool samples but in older (2–5-year-old) stunted and non-stunted children, which may account for no difference in alpha diversity between the stunted and control groups (16). In addition to the low alpha diversity observed in severely stunted children, overall, a higher HAZ was also significantly associated with higher alpha diversity, aligning with the literature suggesting that higher alpha diversity in stool is generally associated with better health outcomes.

We observed a significant difference in beta diversity (using the unweighted unifrac distance metric) between the stunted and normal height groups, similar to other studies (15,18). These findings imply significant phylogenetic differences in the microbial communities between groups and requires further exploration in larger studies of the microbiome in stunting.

As reported in previous birth cohorts, *Actinobacter* was dominant at phylum level in the first few months of life, especially in infants with breastmilk exposure (figure 9, supplementary material), followed by Firmicutes and Proteobacteria (29,30). However, there appears to be a substantially higher relative abundance of *Actinobacter* in this study population when compared with some high income birth cohorts (31). The *Actinobacteria* phylum comprised predominantly *Bifidobacterium* which is known to be present in breastmilk at high abundance, figure 10, supplementary material (21). In addition, breastmilk contains prebiotics in the form of human milk oligosaccharides, that

promote the growth of *Bifidobacterium* (29,32). The high *Bifidibacterium* relative abundance in this study may be due to most children being exposed to some breast milk feeding (72%). The relative abundance of *Bacteroides* in this study was low in the first 6 months and gradually increased with advancing age. Similarly low *Bacteroides* abundance in early life have been described in other African pediatric cohort studies (33). In contrast, cohorts from high-income countries have shown particularly high *Bacteroides* abundance in early life (31). Vatanen et al. described similar findings of higher *Actinobacter* and lower *Bacteroides* abundance in rural, lower SES, Russian infants compared to urbanized, higher SES Finnish and Estonian infants (29). Possible explanations for these differences include the effects of delivery modality, the younger age of this population, geographic location, effects of urbanization and sanitization and/or SES variation in the study populations.

At genus level, *Ruminococcus* had higher relative abundance in the normal height group compared to the stunted group over the first 24 months of life, figure 12, supplementary material. On MaAsLin analysis at genus level, *Ruminococcus* was found to be significantly depleted in the stunted group versus the normal height group (q=0.06), after adjusting for repeated samples, age, method of stool collection, sex, SES, mode of delivery and feeding, figure 7. This feature persisted at species level, with *Ruminococcus gnavus* being significantly depleted in the stunted group compared to the normal height group (q = 0.13), figure 8. *R. gnavus* is classified under the phylum *Firmicutes* and the class *Clostridia*. In the study by Vonasch et al., members of the butyrate producing *Clostridia* were also found to be significantly depleted in the stunted group, compared to controls (16). Butyrate and other short-chain fatty acids (SCFA) are essential to the health of the gut epithelium and gut barrier integrity, and play a role in systemic immune modulation (34,35). Blanton et al. have shown that gnotobiotic mice took on the undernourished phenotype of the donor when inoculated with the microbiome from stunted and acutely wasted children in a Malawian cohort (12). The introduction of *R. gnavus* into the gut microbiome of these humanized mouse models, was capable of ameliorating the growth abnormalities.

The evidence implicating the microbiome in the pathogenesis of childhood stunting is gradually expanding, however, differences in taxonomic composition of the microbiome between stunted and non-stunted children appear to vary from study to study, with some studies suggesting that the functional characteristics of the microbial community may play an important role (19). Further characterization of community composition and functional metabolic potential is essential to understanding variations in the microbiome across geographies and ultimately, necessary for the development of potential microbiome therapeutics. Taken together, the findings from this study

support the growing evidence that important butyrate producing organisms within the class *Clostridia* are depleted in children with stunting. In addition, studies have begun unraveling the underlying causal mechanisms of specific microbes or consortia of microbes, including R. *gnavus*, that could lead to novel approaches to treating stunting with defined microbiome therapies in addition to feeding.

Given that geographical influences of diet and local environmental impact on individual microbial profiles, findings from this study of South African children with undernutrition may not be generalizable to undernourished or stunted children residing in other LMICs. However, studies show that SES and urbanization may have more significant influences on the microbiome than geography alone (36,37). This analysis was performed on a subset of stool samples, from a larger birth cohort, that was originally selected to investigate the role of the microbiome in the development and progression of childhood pneumonia. However, within this study population, there were a substantial number of children with stunting and normal linear growth, which facilitated robust analysis of the microbiome in stunting. A limitation is that 29% of the study population had only 1 stool sample included in the analysis. In addition, 2 different methods of stool sample collection were used in this study which could introduce a sampling bias into the microbiome analysis. To ameliorate the effect of different sampling methods, all multivariate models adjusted for method of sample collection.

CONCLUSION

This is the first South African study to analyze the gut microbiome in longitudinally collected stool samples from children with stunting compared to normal height controls. We found a distinct microbial pattern with significant depletion of a known SCFA-producing organism, *R. gnavus,* in the stunted group compared to children with normal linear growth. Interestingly, the same organism has previously been shown to ameliorate features of undernutrition in animal models. These findings support the growing body of evidence implicating the microbiome in children with stunting and support the rationale for further exploring the potential of microbiome therapeutics to treat stunting. This is particularly relevant in the context of disappointing results from large-scale studies that attempted to address nutritional and environmental exposures in children at risk of stunting (8). Future larger studies should consider deeper longitudinal characterization of the gut microbiome in children across diverse geographies, before and after the development of stunting. These future studies should include metagenomic sequencing of duodenal aspirates, biopsies and stool samples,

stool metabolomics and serological markers of gut barrier disruption. Such studies will help understand the mechanistic role of the microbiome in the pathogenesis of stunting during childhood.

Declarations

Ethics approval

Ethic approvals for this study (739/2016) and the Drakenstein Child Health Study (HREC 149/2013) were obtained from the Human Research Ethics Committees, Faculty of Health Sciences, University of Cape Town. Mothers provided written informed consent at enrolment and annually.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

SB processed and analyzed the clinical and microbiome data, interpreted findings, and drafted the manuscript.
SCW helped process microbiome data and contributed to analysis and significantly contributed to drafting of the manuscript.
TV contributed to microbiome data analysis and interpretation and drafting the manuscript.
MO, MPN, HJZ significantly contributed to drafting of the manuscript.
All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Table 12: Participant characteristics (n=138)

Characteristic	**n (%)**	
Samples (n=401)		
Aspirates	190	(47%)
Whole stool	211	(53%)
Age at stool collection (months)		
0 – 6	192	(58%)
6 – 12	127	(29%)
12 – 24	73	(12%)
>24	9	(1%)
Number of stool samples per participant		
1	40	
2	8	
3	67	
4	2	
≥5	21	
Sex (%)		
Female	62	(45%)
Male	76	(55%)
Mode of delivery (%)		
NVD	113	(82%)
Caesarian	25	(18%)
Socio-economic quartile		
1 - lowest	39	(28%)
2 - low-moderate	51	(37%)
3 - moderate-high	23	(17%)
4 - high	25	(18%)
Birth weight (kg) (mean, SD)	2.9	(0.79)
HIV exposed		
Yes	40	(29%)
No	98	(71%)

NVD: normal vaginal delivery; SD: standard deviation; HIV: Human immunodeficiency virus

Table 13: Participant nutritional status

Anthropometric measure	**n**	**%**
Stool samples associated with stunting (WHZ <-2)		
Yes	104	26%
No	278	69%
Not measured	19	5%
Height (10 months)		
Normal Height	100	72%
Moderately Stunted	15	11%
Severely Stunted	19	14%
Not reported	4	3%
Ever stunted		
Yes	48	35%
Ever severely stunted		
Yes	28	20%

WHZ: weight-for-height z score

FIGURES

Fig. 4: Stool collection time-points

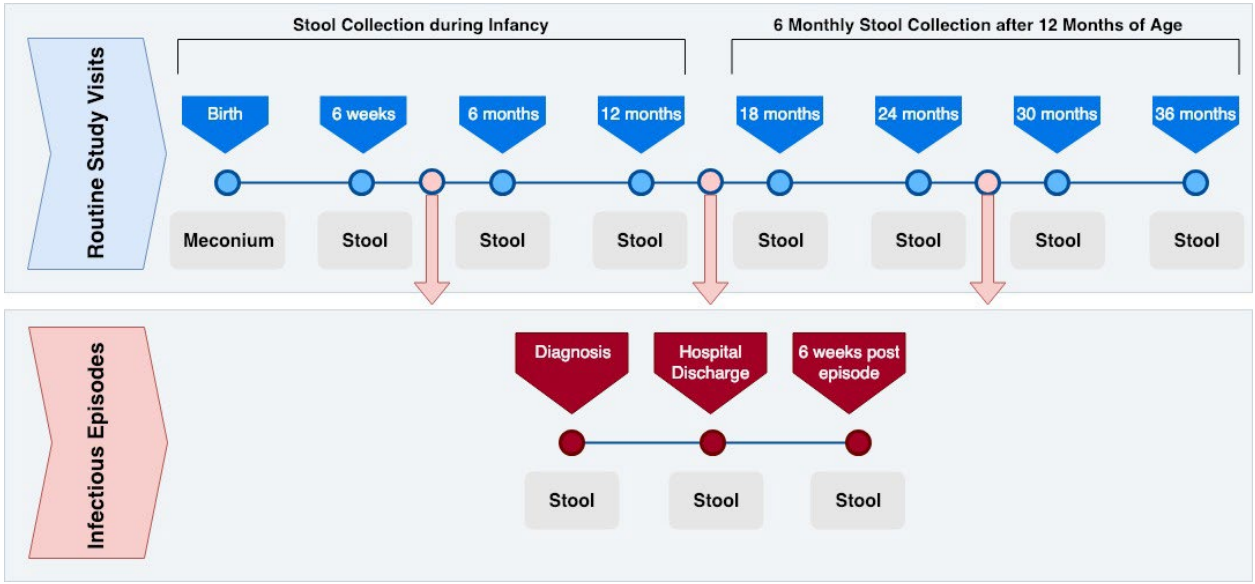
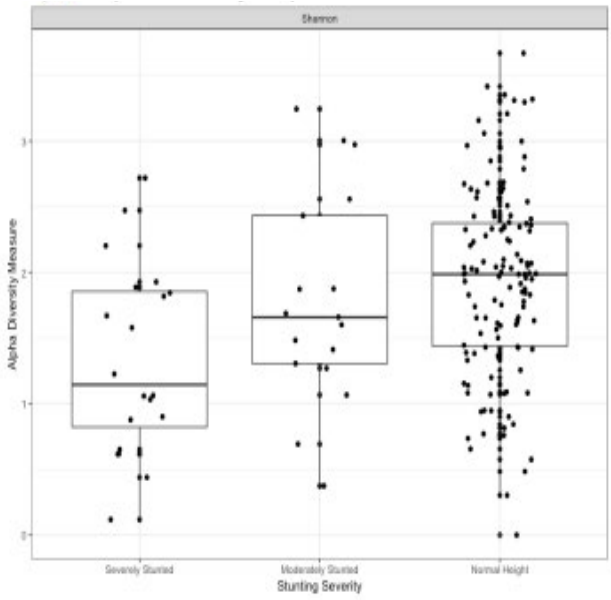


Fig. 4: Stool collection on the Drakenstein Child Health Study. Samples analyzed in this study comprised a subset of longitudinal stool samples collected at both routine study visits (top panel) and samples collected during pneumonia episodes (bottom panel). Stool samples were collected using 2 methods, raw stool collection from the child's diaper or rectal aspirates. The method of collection for longitudinal samples were consistent across all time points for each individual (i.e. all samples from the same participant were either collected as raw stool or rectal aspirate).

Fig. 5: Alpha diversity measures

5A



5B

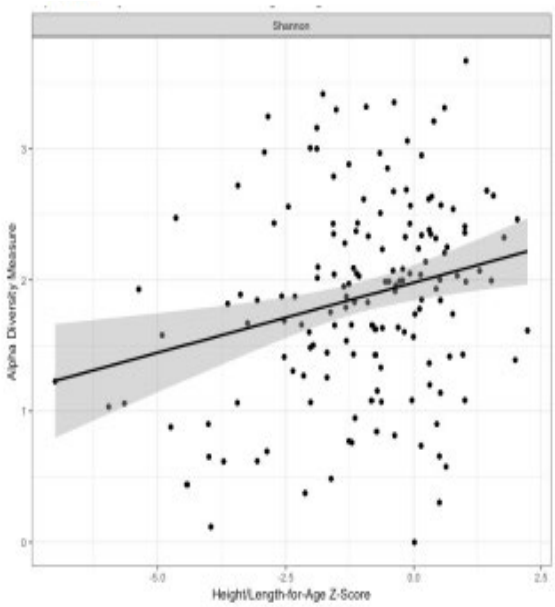


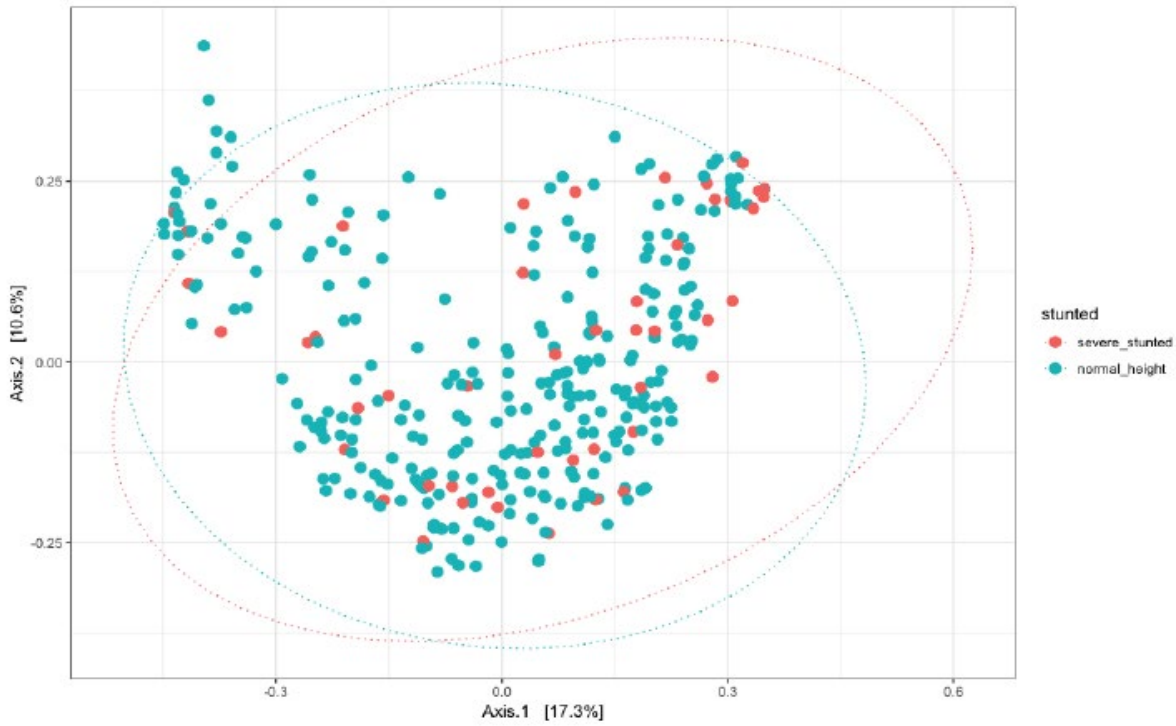
Fig. 5A: Alpha diversity by stunting severity

Figure 5A Alpha diversity (Shannon diversity) was significantly lower in severely stunted children compared to normal height children (p = 0.009) after adjusting for repeated samples, age, sex, mode of delivery, method of stool collection, SES, breastmilk exposure and history of antibiotic use.

Fig. 5B: Alpha diversity by height/length for age z score

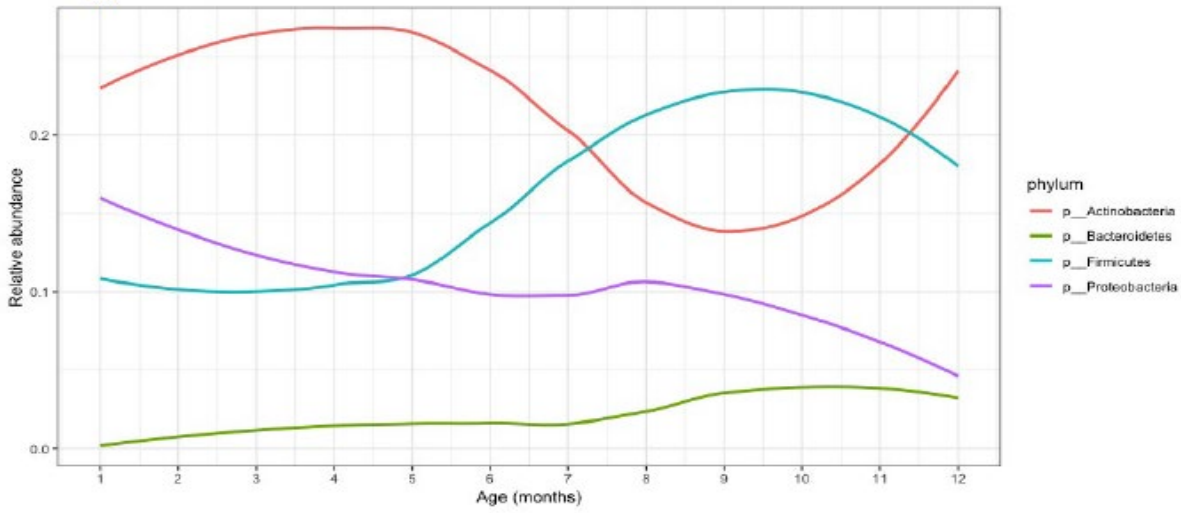
Figure 5B Alpha diversity in was significantly lower in children with lower length-for-age z scores (p=0.002), adjusted for longitudinal sample collection, age and method of collection.

Fig. 6: Beta Diversity (Unweighted Unifrac) between stunting groups



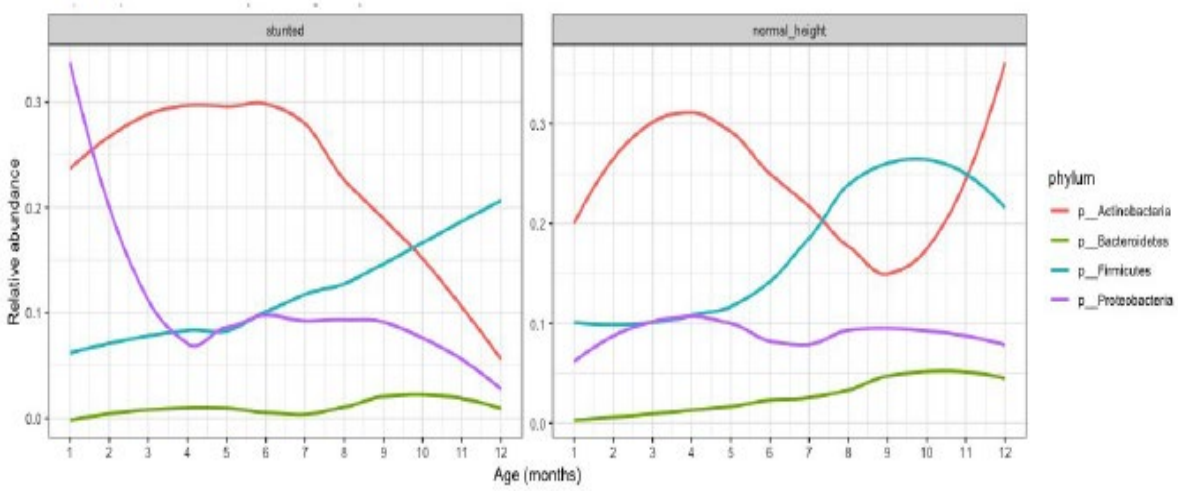
Beta diversity analysis using unweighted Unifrac distances, shows slight separation of samples from severely stunted participants versus normal height participants. On PERMANOVA analysis, samples from severely stunted participants were significantly different compared to those of normal height participants (p=0.001) after adjusting for age, mode of delivery, method of stool collection, breastmilk exposure and antibiotic use.

Fig. 7A: Community composition of all infants at phylum level



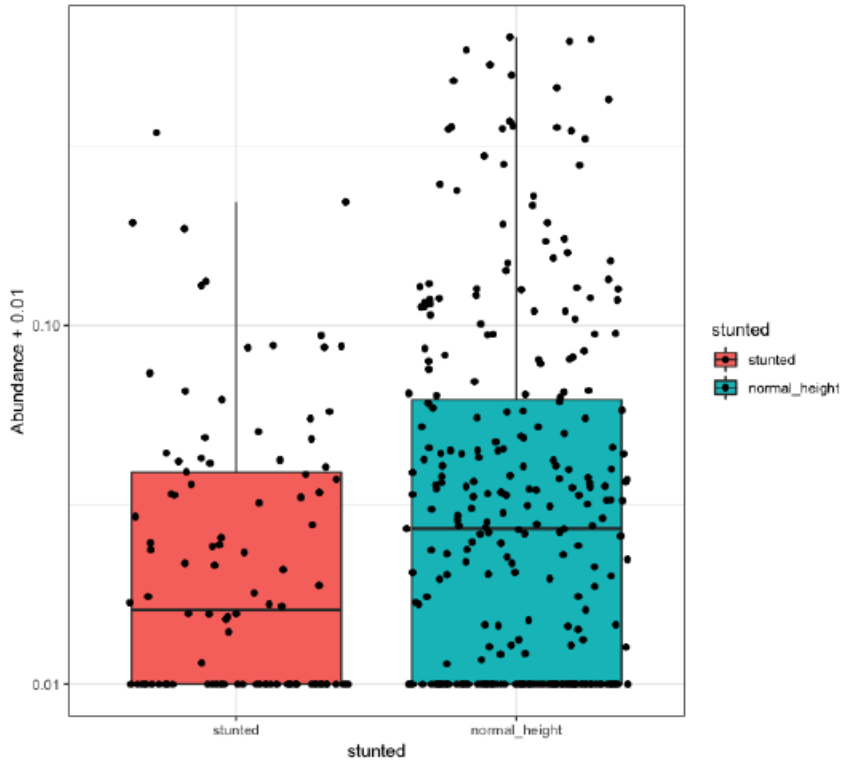
In the first 6 months, *Actinobacter* was dominant at phylum level, which coincides with breastmilk exposure. *Proteobacteria* is dominant early on (first 5 months), thereafter abundance levels decrease progressively. Firmicutes are the third most abundant during the first 5 months, but progressively rise thereafter, to become the most dominant phylum after 6 months. *Bacteroidetes* is under-represented in comparison to the other 3 phyla during the entire first year, with abundance appearing to gradually increase by the end of the first year.

Fig. 7B: Community composition at phylum level stratified by stunting



In stunted children, *Actinobacter* was also dominant at phylum level. Compared to normal height children, the stunted group appear to have a higher *Proteobacteria* abundance in the first 3 months, which then declines to similar levels as the normal height group. *Bacteroidetes* also appears to be depleted in the stunted group relative to the normal height group, but was not statistically significant.

Fig. 8: Species level differential abundance testing between stunted and non-stunted groups shows significantly depleted *Ruminococcus gnavus* in stunted group



On MaAsLiN analysis at species level, the relative abundance of *Ruminococcus gnavus* was found to be significantly depleted in the stunted group compared to the normal height group (q = 0.13) after adjusting for the longitudinal nature of sampling, method of stool collection and participant age.

SUPPLEMENTARY MATERIAL

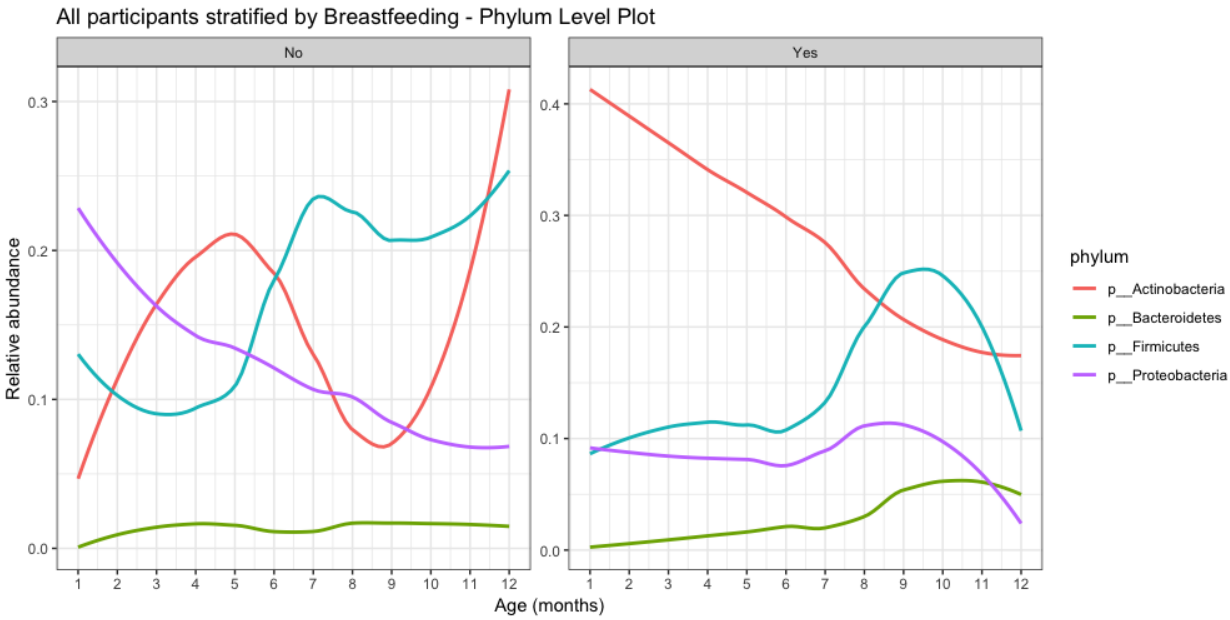
Table 14: Comparison of baseline metadata associated with stool from stunted and non-stunted participants

Characteristic	Stunted samples (n=104)	%	Non-stunted samples (n=278)	%	p-value
Samples (n=382*)					
Aspirates	56	(54%)	124	(45%)	0.13
Whole stool	48	(46%)	154	(55%)	
Mean age at stool collection (months, CI)	9.3	(-0.49 – 2.77)	8.1	(-0.49 – 2.77)	0.17
Sex (%)					
Female	43	(41%)	135	(49%)	0.25
Male	61	(59%)	143	(51%)	
Mode of delivery (%)					
NVD	77	(74%)	240	(86%)	<0.05
Caesarian	27	(26%)	38	(14%)	
Socio-economic quartile					
1 - lowest	34	(33%)	84	(30%)	0.07
2 - low-moderate	44	(42%)	94	(34%)	
3 - moderate-high	9	(8%)	54	(19%)	
4 - high	17	(17%)	46	(17%)	
HIV exposed					
Yes	45	(43%)	209	(75%)	<0.05
No	59	(57%)	69	(25%)	
Preterm (<37 weeks GA)					
Term	50	(48%)	232	(83%)	<0.05
Pre-term	54	(52%)	44	(17%)	
On antibiotics at stool collection					
Yes	11	(11%)	32	(12%)	0.94
No	93	(89%)	246	(88%)	
Ever breastfed					
Yes	73	(70%)	204	(73%)	0.62
No	31	(30%)	74	(27%)	

*19 participant height data-points missing

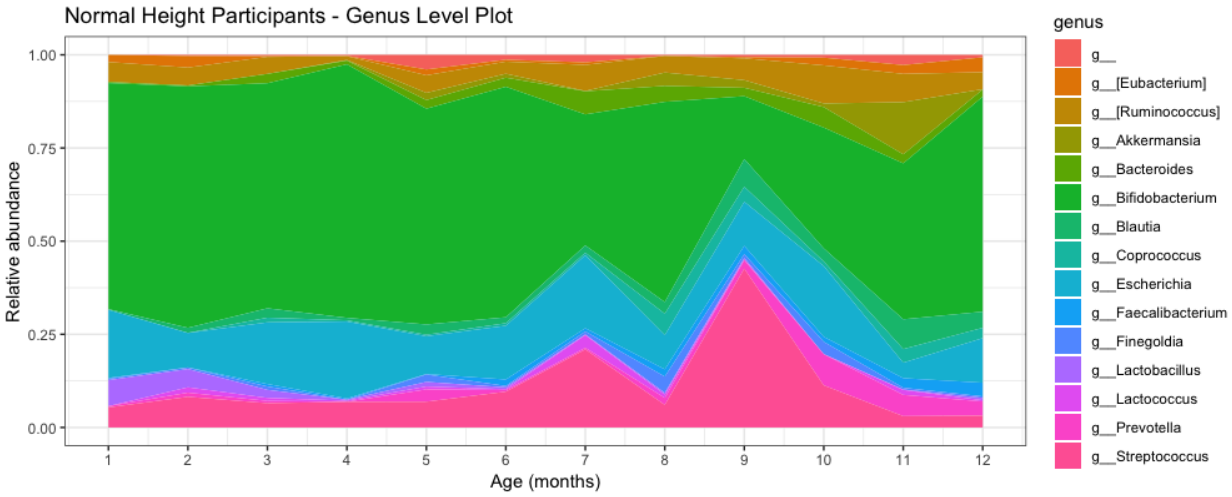
CI: Confidence interval; NVD: normal vaginal delivery; HIV: Human immunodeficiency virus; GA: gestational age

Fig. 9: Phylum level composition in participants who were exposed to breast milk compared to never exposed



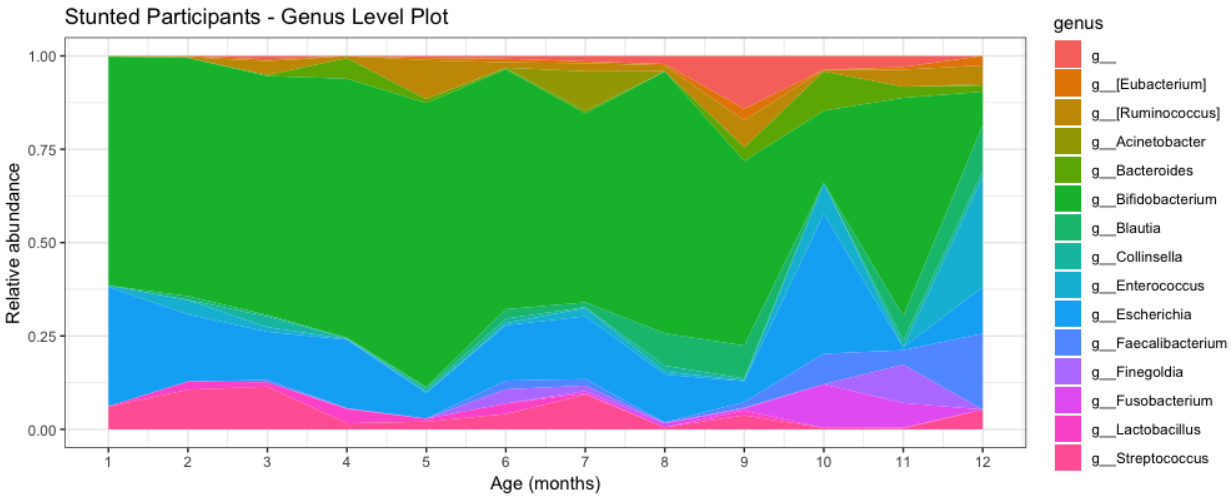
Higher levels of *Actinobacter* are present in participants who were ever exposed to breastmilk compared to those who were never exposed to breastmilk.

Fig. 10: Community composition of normal height children at genus level



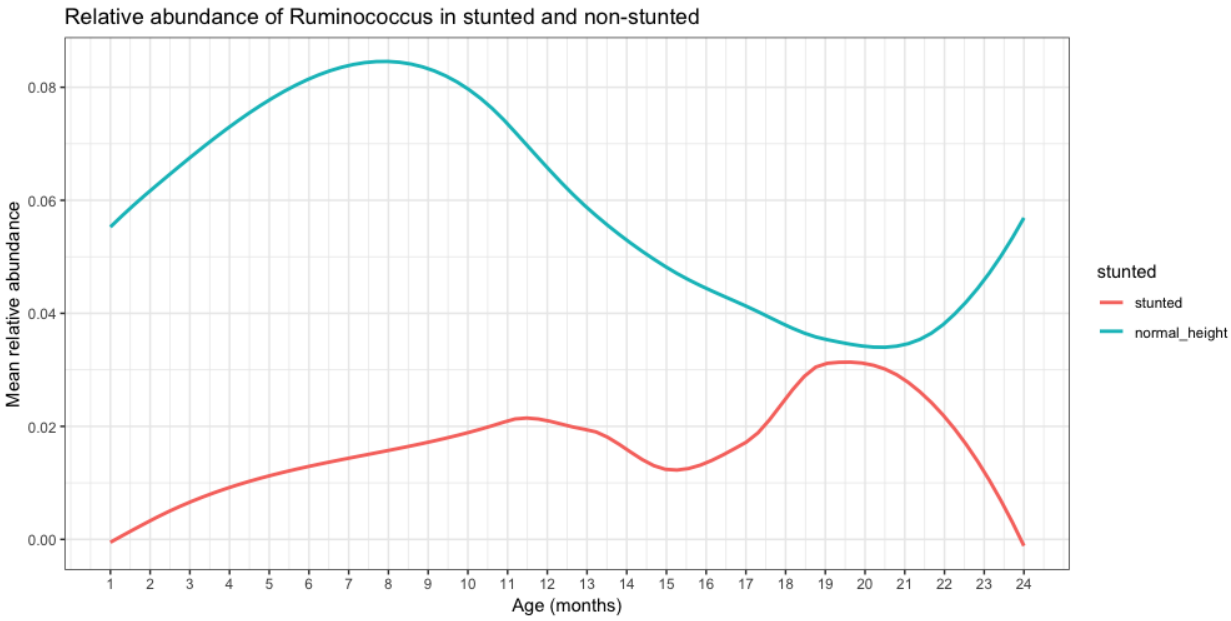
At Genus level in normal height children, *Bifidobacterium* dominates during the first 6 months. *Ruminococcus*, is detectable within the first month and appears to gradually increase with advancing age in these healthy children.

Fig. 11: Community composition of stunted children at genus level



At Genus level, in the stunted group, *Bifidobacterium* is dominant. *Ruminococcus* was found to be significantly depleted in the stunted children comparison to the normal height group (q= 0.03).

Fig. 12: *Ruminococcus* abundance in stunted versus non-stunted over first 2 years



The genus *Ruminococcus* is significantly lower in the stunted group versus the non-stunted group from birth to 24 months.

Chapter 5: Gut Microbiome Dysbiosis Precedes Childhood Pneumonia: Findings from a South African Birth Cohort

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Abstract

Importance: Childhood pneumonia is a leading cause of under-5 mortality. Evidence linking the gut microbiome to lung health, termed the 'gut-lung-axis' comes primarily from preclinical studies with few cross-sectional human studies. No studies have investigated the gut microbiome longitudinally, including prior to pneumonia onset, and none from low-middle income (LMIC) settings where disease burden is highest.

Objective: To evaluate longitudinal microbiome changes in children with pneumonia in a LMIC and to determine microbial factors associated with the risk of developing pneumonia.

Design, Setting and Participants: Children aged 1 to 40 months from a South African birth cohort.

Exposure: WHO defined pneumonia.

Main Outcomes and Measures: Stool microbiome composition by V4 16S rRNA amplicon sequencing during pneumonia episodes.

Results: In total, 136 participants (mean age 8.5 months) were included, 90 with one or more pneumonia episodes and 46 non-pneumonia controls. Alpha diversity was significantly higher among non-pneumonia controls compared to the pneumonia group (p=0.01), however, no difference was observed between preceding, episode, and follow-up samples. Beta diversity was significantly different between pneumonia vs non-pneumonia samples (p<0.001) and between pneumonia timepoints (p=0.017). MaAsLin analysis of preceding, episode and follow-up time points, adjusting for the method of stool collection, age, and antibiotic exposure showed significant enrichment in Proteobacteria (q=0.11), specifically *Escherichia coli* (q=0.23), during pneumonia episodes. During pneumonia episodes, *Enterococcus, Porphyromonas and Campylobacter* were significantly enriched (q=0.14, q=0.02, q=0.17) and Eubacterium and *Oscillospira* were significantly depleted (q=0.17, q=0.17). In a robust mixed-effects model adjusted for repeat samples, method of stool collection, age, antibiotic exposure, mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, nutritional status and daycare exposure, *E. coli* was significantly enriched in samples preceding pneumonia episodes compared to age-matched non-pneumonia samples (q=0.04).

Conclusion and Relevance

In this first longitudinal study of the gut microbiome in LMIC children with pneumonia, we showed dysbiosis during pneumonia episodes characterized by expansion of *E. coli*, and depletion of short chain fatty acid-producing microbes. Dysbiosis was present prior to the onset of pneumonia,

characterized by expansion in *E. coli*, supporting the gut-lung axis concept that gut microbiome dysbiosis is a risk factor for pneumonia.

Background

Childhood pneumonia is the leading infective cause of death in children under 5 years globally accounting for 0.54 million deaths in children aged 1-59 months in 2019 (1). While global incidence and mortality have reduced over the past decade, the burden of disease remains high especially in low-middle income countries (LMICs), including sub-Saharan Africa (2,3). In high income countries, childhood pneumonia is one of the most common causes of hospitalization, placing a significant burden on healthcare systems (2,4). Therefore, exploring new strategies to prevent pneumonia during early childhood is imperative.

The gut microbiome has emerged as a potential therapeutic target for several diseases (5,6). The microbiome is considered an 'organ system' contributing to essential metabolic processes in humans, and disruption is associated with both infective and chronic inflammatory diseases (7). Growing evidence supports the role of the gut microbiome in the development of lung immunity and protection against respiratory tract infections (RTI), termed the 'gut-lung' axis. Several preclinical studies have demonstrated aberrant lung immune function and increased susceptibility to RTI in the context of a dysbiotic gut microbiome (8,9). Additionally, microbial metabolites such as desaminotyrosine and short-chain fatty acids (SCFA) produced by gut bacteria have been shown to enter the circulation, stimulating immunity against viral RTI (9,10).

Few cross-sectional studies, primarily in high-income settings, have described a dysbiotic gut microbiome in children during pneumonia episodes, compared to non-pneumonia controls, suggesting that RTI itself may further disrupt the healthy microbiome (11). The gut-lung axis is bidirectional with several mechanisms contributing to the disruption of the gut microbiome during a pneumonia episode (9). Deciphering cause versus effect from cross-sectional data is challenging.

Longitudinal studies provide deeper insight into temporal changes in the microbial community and help distinguish cause versus effect. However, to date, no studies have evaluated the microbiome longitudinally, before, during, and after a pneumonia episode. Additionally, adjusting for multiple microbiome perturbations during childhood (e.g., mode of delivery, prematurity, age, infant feeding, antibiotics), is important during analysis, and has been limited in most studies (12–14). A meta-data-rich birth cohort addresses these limitations and facilitates the analysis of the microbiome preceding the onset of pneumonia.

This study aimed to evaluate the gut microbiome longitudinally in children before, during, and after a pneumonia episode, compared to non-pneumonia controls.

Methods

Study design and patient population

The Drakenstein Child Health Study (DCHS) is a birth cohort located in a peri-urban setting in South Africa. Mothers, 18 years and older and in their second trimester of pregnancy were enrolled from two public health clinics and their children were followed up from birth through 10 years (study is ongoing). During the first year, routine study visits occurred at birth, 6, 10, 14 weeks, 6 months, and 12 months; followed by 6-monthly visits after 12 months. Between birth and 2 years, children were actively followed for the development of pneumonia at community-based clinics and the single referral hospital servicing this geographic region. WHO criteria were used to define pneumonia and participants were assessed by trained clinical staff during each ambulatory or hospitalized episode.

Risk factors for the development of pneumonia that were also relevant to microbiome perturbation included birth weight, gestational age, mode of delivery, prematurity, sex, infant feeding method, age at stool collection, day-care exposure and HIV exposure or infection were collected (9). Antibiotic exposure was defined as any antibiotic exposure during the first year of life. Severity of pneumonia episodes; antibiotics exposure at the time of stool collection; and any antibiotic exposure during the pneumonia episode were also collected.

Based on the South African Extended Programme on Immunisation schedule vaccinations were given at birth, 6-, 10-, 14-weeks, 6-, 9- and 18 months. Scheduled vaccines included the 13-valent pneumococcal vaccine, rotavirus vaccine, and vitamin A supplementation per national policy (15).

A subset of participants with one or more pneumonia episodes on the DCHS, between birth and 3 years of age, was identified for this analysis based on the availability and method of stool collection preceding, during, and after their pneumonia episode. Non-pneumonia controls were a subset of children on the DCHS between birth and 2 years with no reported pneumonia or wheezing episodes, who had existing gut microbiome 16S rRNA gene amplicon sequencing performed at routine study visits as part of a separate sub-study. Non-pneumonia controls were selected from this existing dataset based on age to match the age of participants with pneumonia at the time of preceding sample collection.

Stool sample collection

Due to the challenges associated with stool collection in pediatric populations, samples were either collected from the diaper (passed stool) or as a rectal aspirate (aspirate). Mothers collected a stool sample from the diaper at home on the morning of their routine study visit at birth, 6 weeks, 6-, 12-, 18-, 24-, 30- and 36-months. Stool samples collected at home were transported in a cooler to the study site. Aspirated stool samples were collected by trained staff under standard-operating procedures at the study site by aspirating stool through a rectal tube. Given the two methods of collection, all microbiome analyses were adjusted for this variable.

During a pneumonia episode, participants had a stool sample collected at the time of diagnosis ('episode') and 4 to 6 weeks after the diagnosis ('follow-up'). For this analysis, a preceding sample ('preceding') was identified as the most recent stool sample collected prior to the pneumonia diagnosis. Therefore, most pneumonia episodes have a triad of samples associated with the episode. Importantly, in this analysis, only those samples for which the same method of collection was used for the pneumonia episode, preceding, and follow-up timepoints were included.

Figure 13 shows the triad of samples associated with each episode, the age category of participants and the method of collection at each episode.

Microbiome sequencing

Microbiome sequencing procedures on the DCHS were previously described elsewhere (16). Briefly, stool samples were thawed, and DNA extraction was done using QIAsymphony DSP Virus/Pathogen Mini Kit® (Qiagen GmbH, Hilden, Germany). Thereafter, sequencing of the V4 region of the 16S rRNA gene was performed on an Illumina® MiSeqTM platform.

Fastq files generated from the Illumina platform were further processed using QIIME2 and amplicon sequence variants (ASVs) generated (17). Taxonomic identification was performed using Greengenes in QIIME2 (18). Further analysis was performed using the Phyloseq package (Version 1.42.0), in R (Version 4.2.2).

Statistical analysis

Continuous clinical variables were reported as either mean or median. Categorical data were reported as proportions.

Alpha diversity was measured using the Shannon index. Significant differences in alpha diversity were assessed using a mixed effects model adjusting for age and method of stool collection.

Beta diversity was measured using unweighted Unifrac distance. Statistical significance was assessed by Permutational multivariate analysis of variance (PERMANOVA), adjusting for age and method of stool collection.

The taxonomic composition was displayed as bar plots showing the relative abundance of taxa at phylum and genus levels. Aggregated taxonomic composition plots display the mean abundance of taxa by clinical covariates.

Differential abundance by clinical covariates was determined using the microbiome multivariate association with linear models (MaAsLin2) package in R (Version 1.12.0). All statistical models were adjusted for repeat sampling, age, method of stool collection and antibiotic exposure at the time of stool collection. FDR-adjusted p-values (q-values) were determined with significance being a q-value < 0.25.

Results

Demographics

A total of 136 participants were included, 90 with one or more pneumonia episodes and 46 non-pneumonia controls. Mean age was 8.5 months (sd=6.4, range=0-40 months) with a slightly higher mean age in the pneumonia compared to non-pneumonia group (Table 15). Sex distribution, HIV exposure, vaccination rates and duration of exclusive breastfeeding were similar across groups (Table 15). A higher proportion of children in the pneumonia group had lower household incomes (<$67-$333 per month) and were born prematurely compared to the non-pneumonia group. A higher proportion of children in the non-pneumonia group were born by caesarian section. Stunting rates were similar across groups.

There were 116 pneumonia episodes among 90 participants in the pneumonia group. Most were ambulatory pneumonia episodes with only 10% being hospitalized episodes. A quarter of participants were on antibiotics at the time of the pneumonia episode stool sample collection (23%) whereas 82% received antibiotics after collection of the pneumonia episode stool sample (82%). The most frequently administered antibiotic was oral amoxicillin. More than a third (39%) of participants had recurrent pneumonia episodes.

Diversity

In a mixed-effects model of alpha diversity, participants' age (<6 months, 6-12 months, and >12 months), and their pneumonia group (pneumonia vs non-pneumonia) were the most significant determinants of alpha diversity, after adjusting for the method of stool collection, mode of delivery,

exclusive breastfeeding, sex, prematurity, HIV exposure, daycare exposure and antibiotic exposure (Figure 14A, 14B). Participants' alpha diversity significantly increased with age (<6 vs 6–12 months: p<0.001; 6-12 vs >12 months: p<0.001, Figure 14A). Alpha diversity was significantly higher among the non-pneumonia samples compared to pneumonia samples (preceding, episode and follow-up samples) despite the non-pneumonia group being younger (p=0.01).

No difference in alpha diversity was observed between the preceding, episode, and follow-up samples (Figure 17A, supplementary material). Among all samples in the pneumonia group, alpha diversity was significantly lower in those exposed to antibiotics at the time of stool sample collection after adjusting for stool collection method and age (p=0.031, Figure 17B, supplementary material).

On beta diversity analysis among all participants, there was significant clustering by age and pneumonia group (pneumonia vs non-pneumonia), adjusted for the method of stool collection, mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, daycare exposure and antibiotic exposure (p=<0.001 and p=<0.001 respectively, Figure 14 C, D). Within the pneumonia group, there was significant separation of the preceding, episode, and follow-up samples (p=0.017), adjusted for covariates above (Figure 18, supplementary material).

Taxonomic composition

Phylum level

Across the 3 age categories (birth-6 months, 6-12 months, >12 months) at a phylum level, the microbial community was dominated by Actinobacteria followed by Firmicutes with a much lower abundance of Proteobacteria, Bacteroidetes, and other phyla (Figure 19, supplementary material). Within the pneumonia group, the mean abundance of Proteobacteria was higher in episode samples compared to the preceding samples (Figure 15A, B). Actinobacteria was lower in follow-up samples compared to preceding and episode samples (Figure 15B). Compared to pneumonia samples, non-pneumonia samples had a higher abundance of Actinobacteria, and a lower abundance of Proteobacteria and Firmicutes (Figure 15B).

At a genus level, *Bifidobacterium* was the dominant genus, followed by *Escherichia (E. Coli)* and *Streptococcus* (Figure 15C). *E. Coli and Streptococcus* abundance was at its highest in pneumonia episode samples compared to preceding and follow-up samples (Figure 15D). *Bifidobacterium* abundance appears lowest in follow-up samples compared to episode and preceding samples (Figure 15D).

Significant changes in the microbial community during pneumonia episodes

At a phylum level, the mixed effects MaAsLin analysis, adjusted for repeat samples, method of stool collection, age, and antibiotic exposure showed that Proteobacteria was significantly enriched in pneumonia episode compared to follow-up samples (q=0.11) (Figure 16A). This was not observed between the preceding and episode samples. Furthermore, Actinobacteria was significantly higher in the preceding compared to episode samples (q=0.18). Pneumonia samples also had a significantly higher abundance of Proteobacteria and Firmicutes compared to non-pneumonia samples (q=0.08 and q=0.12, respectively).

At a genus level, in a similarly adjusted model, there was significant enrichment of *E.coli* and *Enterococcus* in pneumonia episodes compared to follow-up samples (q=0.23 and 0.14 respectively) (Figure 16B, Figure 20A, supplementary material). *Porphyromonas* was significantly enriched during pneumonia episodes compared to preceding and follow-up samples (q=0.07 and q=0.02). *Campylobacter* was significantly enriched in episode compared to preceding samples (q=0.17, Figure 20B, supplementary material). *Oscillospira* was depleted in episode compared to preceding samples (p=0.17) and *Eubacterium* was significantly depleted during episode compared to preceding and follow-up samples (preceding vs episode, q=0.17; follow-up vs episode, q=0.20, Figure 20C, supplementary material).

Due to mean age of non-pneumonia controls being lower than the pneumonia group, only age-matched pneumonia samples were included in the comparison of preceding and pneumonia episode samples to non-pneumonia controls (pneumonia group, n=62 versus non-pneumonia group, n=46). In this robust mixed-effects model adjusted for the method of stool collection, age, antibiotic exposure, mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, nutritional status (stunting) and daycare exposure, *E. coli* was significantly enriched in samples preceding a pneumonia episode compared to age-matched non-pneumonia samples (q=0.04, Figure 16C). In a similarly adjusted model, *E. coli* remained significantly enriched in pneumonia episode samples compared to age-matched non-pneumonia samples (q=0.005).

Discussion

This novel longitudinal analysis of the gut microbiome in South African children, showed significant changes in the microbial community, notably, expansion of Proteobacteria, specifically *E. coli*, and reduction in butyrate producers (*Eubacterium and Oscillospira*), during pneumonia episodes compared to preceding and follow-up time points. Additionally, there was pneumonia-associated dysbiosis characterized by higher abundance of *E. coli* in samples taken during a pneumonia episode. Strikingly, dysbiosis preceded the onset of pneumonia clinical symptoms, illustrated by significant enrichment of *E. coli* compared to age-matched non-pneumonia controls.

There is a significant under-representation of African microbiome studies (19). Additionally, there are no studies evaluating the microbiome longitudinally in children preceding, during and following a pneumonia episode. Overall microbiome characteristics in this cohort were similar to other African pediatric studies (20). Alpha diversity progressively increased with age and clustering by age group on beta diversity (Figure 14A, 15A), attributed to maturation of the infant microbiome, changes in early life diet, and the shifts from an aerobic to an anaerobic intestinal environment (20–22). During the first 12 months, the microbiome was dominated by Actinobacteria, specifically *Bifidobacterium*, driven primarily by breastmilk exposure (23). Similar to other pediatric studies, Proteobacteria, specifically *E. coli,* was dominant in the first few months following birth. This typically declines after 6 months; however, the persistently high abundance of *E. coli* well beyond the first 6 months in this population, suggests an ongoing dysbiosis in this population (21,22).

Cross-sectional studies have demonstrated dysbiosis during pneumonia episodes (9,24). Several studies showed decreased alpha diversity and enrichment in Proteobacteria during pneumonia episodes (9,24). This robust analysis, adjusted for multiple microbiome confounders, showed similar findings including lower diversity and significant expansion in Proteobacteria, specifically, *E. coli*, in pneumonia episode samples compared to controls (Figure 14B, 16B). Animal studies showed an increase in *E. coli* abundance in the absence of antibiotic exposure following infection with influenza (25,26). A study of hospitalized adults with influenza similarly showed higher abundance of *E. coli* in the non-antibiotic exposed group compared to controls (27). Contrary to a case-control study from the U.S. which observed higher prevalence of bronchiolitis in infants with Bacteroides-dominant microbial profiles, no association with Bacteroides was found, possibly due to lower abundance of Bacteroides frequently observed in African pediatric populations (13,28).

The significant difference in beta diversity between preceding, episode, and follow-up time points suggests large shifts in microbial community composition. Longitudinal comparison of community composition at genus level, before, during and after pneumonia episodes showed expansion of *E. coli* during the episode that significantly decreased with resolution of the episode. These novel findings suggest significant perturbation of the gut microbial community characterized by expansion of gram-negative microbes during pneumonia episodes, not attributable to antibiotic exposure, that improves within 4–6-weeks following the episode. The ‘gut-lung axis’ is bidirectional, with several mechanisms contributing to alteration of the gut microbiome composition during RTI, including but not limited to, upregulation of inflammatory cytokines that promote blooms in Proteobacteria (9). Additionally, there was significant enrichment of *Enterococcus* during pneumonia episodes, a finding previously described in cross-sectional studies of children with RSV infection (12).

Characterization of the gut microbiome preceding pneumonia episodes provides unique insight into microbial community changes prior to onset of pneumonia and has not been previously described in the literature. Our findings highlight a shift in community composition from preceding samples to a more dysbiotic composition in pneumonia episode samples. Specifically, significant reduction in SCFA producing microbes (*Eubacterium and Oscillospira*) and significant enrichment in pathogenic gram-negative organisms (*Campylobacter and Porphyromonas*) (19–31). Studies have described the importance of SCFA as part of the gut-lung axis and in the pathogenesis of infective lung disease, including depletion in SCFA-producing organisms during pneumonia episodes (9,11,24). SCFA modulate pulmonary immune function including enhanced pulmonary macrophage and CD8 T-cell effector function vital to viral clearance (9,24,26).

Comparison of the microbiome preceding pneumonia episodes to age-matched, non-pneumonia controls facilitated identification of potential microbial biomarkers that may be associated with risk of developing pneumonia during childhood. At genus level, *E. coli* abundance was significantly higher in samples preceding the onset of a pneumonia episode compared to age-matched, non-pneumonia controls (q=0.04). These findings suggest that compared to healthy, age-matched, non-pneumonia controls, microbiome dysbiosis was present prior to onset of pneumonia symptoms, characterized by asymptomatic bloom in *E. coli*. These findings support the 'gut-lung-axis' thesis, that gut microbiome dysbiosis is an important risk factor in the pathogenesis of RTI during childhood. While animal studies have demonstrated a mechanistic connection between a gut microbiome, lung immunity, and risk for RTI, human studies are lacking due to the paucity of cohort studies evaluating the gut microbiome longitudinal during a RTI.

Limitations of this study include the use of two methods of stool collection due to challenges associated with the collection of stool samples in children. To minimize the effect of the method of sample collection, this analysis only included triads of samples (preceding, episode, and follow-up) that were collected using the same method (i.e., all 3 samples were either rectal aspirates or passed stool) and all statistical analysis adjusted for method of sample collection. A major strength is that it is the first study to report that analysis of longitudinal microbiome sampling surrounding pneumonia episodes in African children.

This longitudinal gut microbiome study preceding, during and after pneumonia episodes demonstrated dysbiosis during the episode characterized by the expansion of *E. coli* and other gram-negative pathogens, and depletion of SCFA-producing microbes. This dysbiosis improved within 4-6 weeks following the episode with a significant reduction in *E. coli*. Our findings also suggest dysbiosis prior to onset of pneumonia episodes, supporting the gut-lung axis concept. Future studies should

include larger sample sizes and evaluate the functional metabolic changes in the gut microbiome using metagenomic sequencing. Generation of further cross-sectional and longitudinal microbiome data will inform the development of future microbiome therapeutics aimed at correcting underlying dysbiosis thereby reducing the risk of developing pneumonia.

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Table 15: Characteristics of participants

Characteristics	Children with pneumonia (n=90)	Non-Pneumonia Controls (n=46)
Age (months) (mean, SD)	8.9 (6.6)	5.6 (3.5)
Sex - Female	40 (44%)	22 (48%)
Household income		
• <R1000 (US$67)	28 (32%)	10 (22%)
• R1000-5000 (US$67-333)	34 (38%)	16 (34%)
• R5000-10 000 (US$333-667)	13 (13%)	10 (22%)
• >R10 000 (US$667)	15 (17%)	10 (22%)
Mode of birth		
• Normal Vertex Delivery	76 (84%)	35 (76%)
• Caesarean section	14 (16%)	11 (24%)
Gestational age (weeks)	37.3	39.1
Premature (<37 weeks)	28 (31%)	4 (9%)
HIV exposed uninfected	27 (30%)	13 (28%)
Exclusive Breastfeeding at least 5 months	14 (16%)	7 (15%)
Nutritional status (stunted)	18* (20%)	10 (22%)
Daycare exposure at 12 months	13 (14%)	9 (20%)
Vaccinations completed	87 (97%)	44 (96%)
Pneumonia events (n=116)		
• Hospitalized	11 (10%)	NA
• Ambulatory	105 (90%)	NA
• Recurrent	45 (39%)	NA
• On antibiotics at stool collection	27 (23%)	NA
• Any antibiotic exposure during pneumonia	74 (82%)	NA

*Proportion of children stunted at the time of pneumonia episode

Figure 13: Sample collection around pneumonia episodes

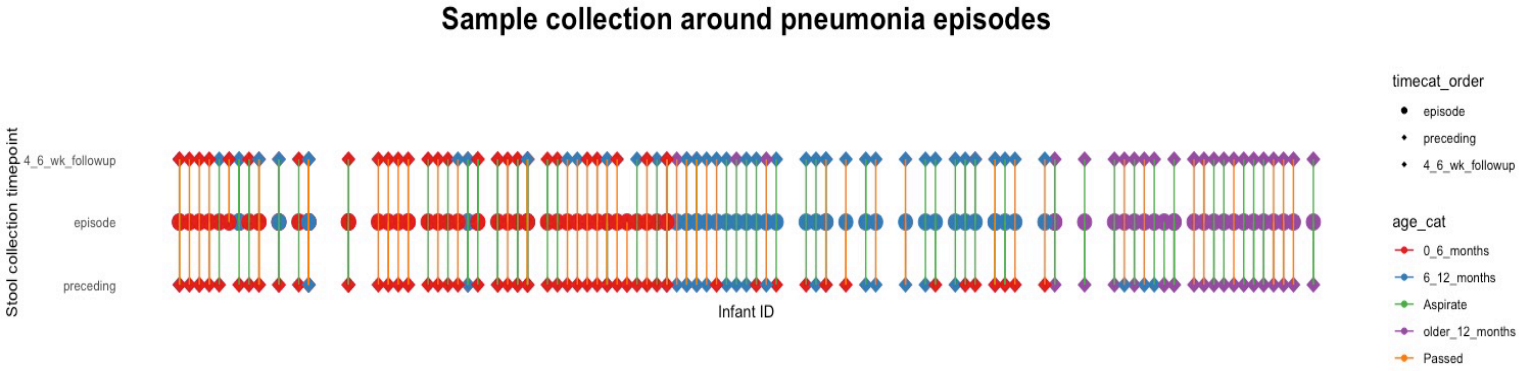
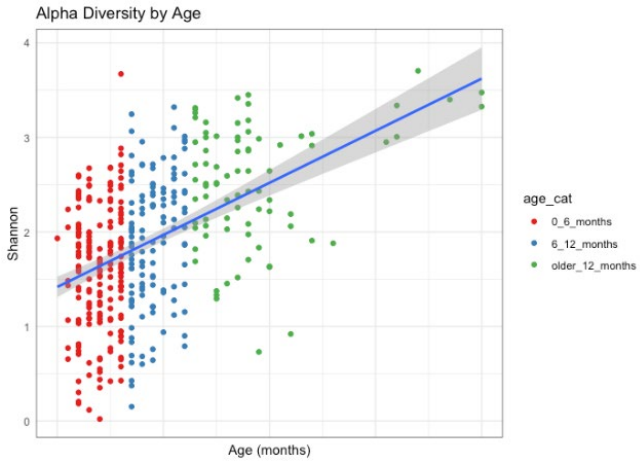


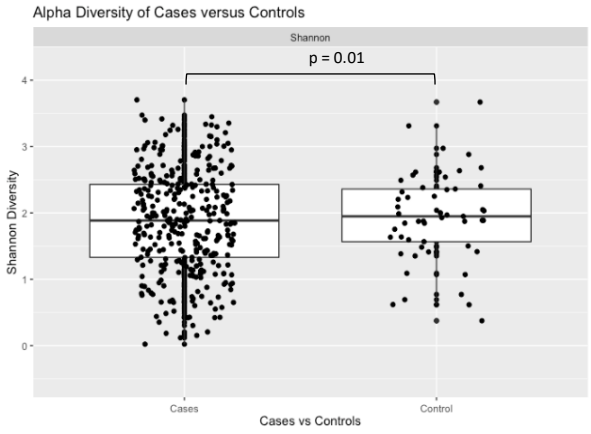
Figure 13: Stool sample collection, showing age distribution and method of sample collection (rectal tube aspirate versus passed stool)

Figure 14: Alpha (Shannon) and Beta Diversity (unweighted Unifrac) across groups

14A



14B



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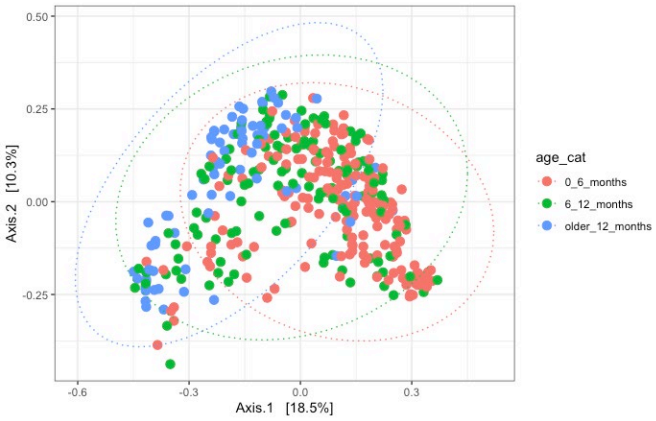


14D

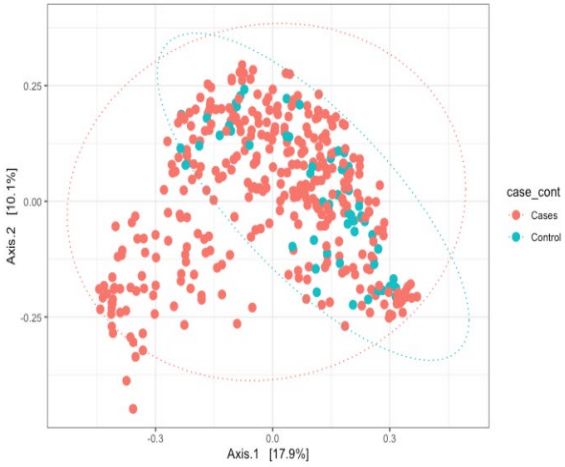
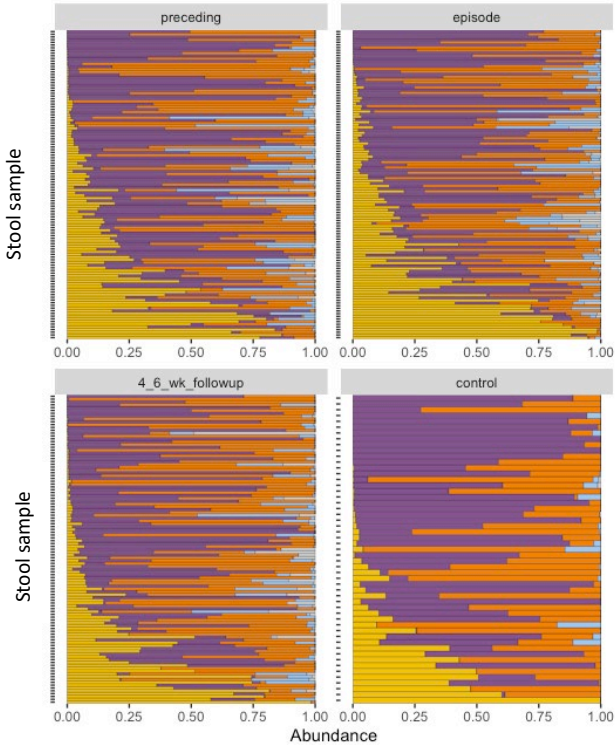


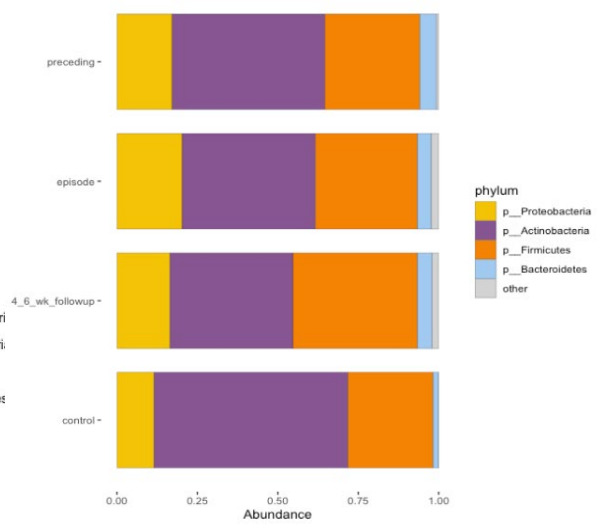
Figure 14: A: Alpha diversity mixed effects model adjusted for method of stool collection (passed vs aspirated), mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, daycare exposure and antibiotic exposure. A: significantly increased if alpha diversity with age. B: Significantly higher diversity in non-pneumonia controls compared to pneumonia cases. Beta diversity PERMANOVA model adjusted for the method of stool collection, mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, daycare exposure and antibiotic exposure. C: Significant separation by age group (PERMANOVA, p=<0.001) D: Pneumonia group clusters separately from non-pneumonia group (PERMANOVA, p=<0.001).

Figure 15: Taxonomic composition at phylum and genus levels surrounding pneumonia episodes versus non-pneumonia controls

15A



15B



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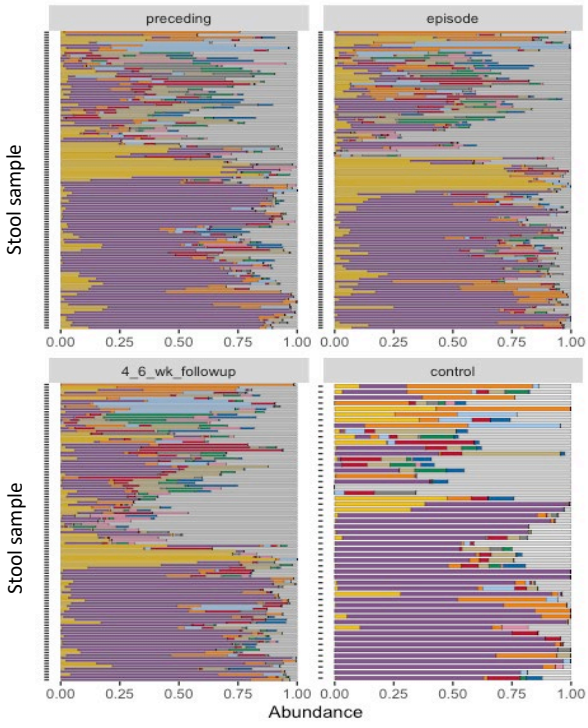

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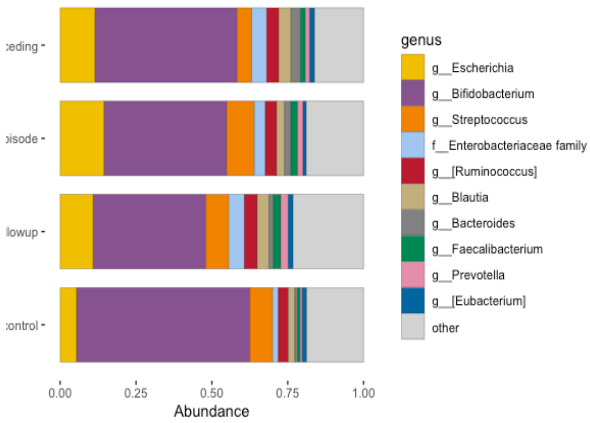
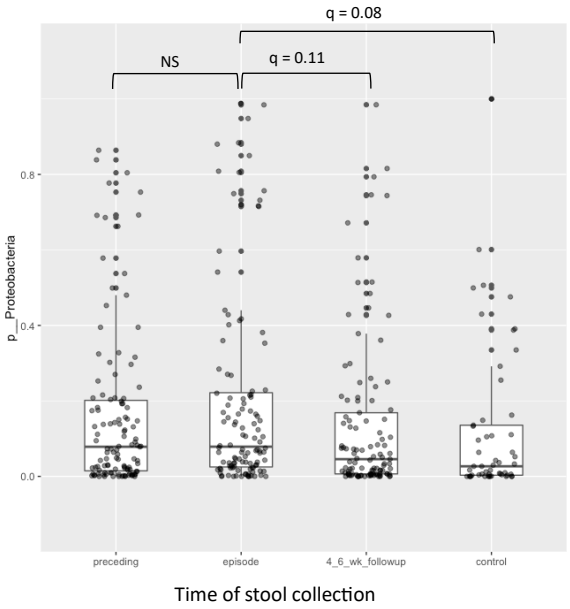


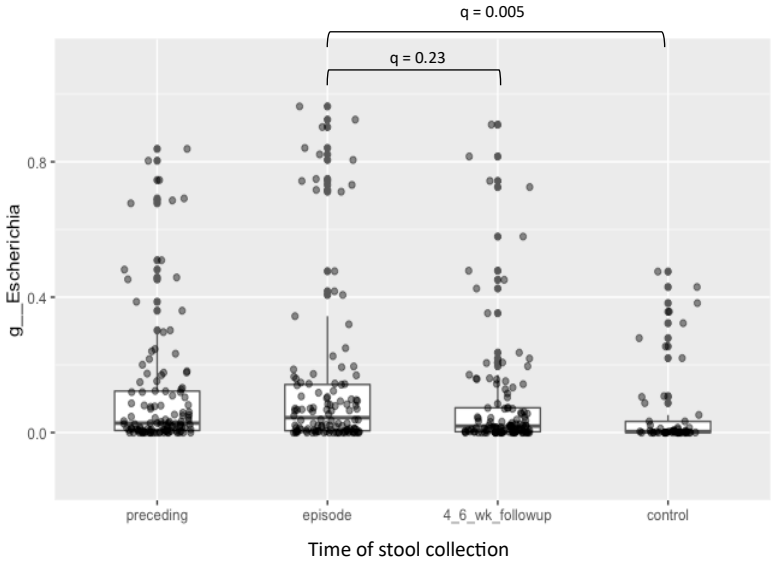
Figure 15: A: Taxonomic relative abundance at phylum level during pneumonia (preceding, episode and follow-up) and in controls. B: Aggregated taxonomic abundance at collection time points during a pneumonia episode compared to controls. C: Taxonomic relative abundance at genus level during pneumonia (preceding, episode and follow-up) and in controls. D: Aggregated taxonomic abundance at collection time points during a pneumonia episode compared to controls. The relative abundance of E. coli appears highest at the time of pneumonia episode.

Figure 16: MaAsLin2 analysis showing significant differentially abundant taxa between pneumonia time points and pneumonia timepoints versus non-pneumonia controls

16A



16B



16C

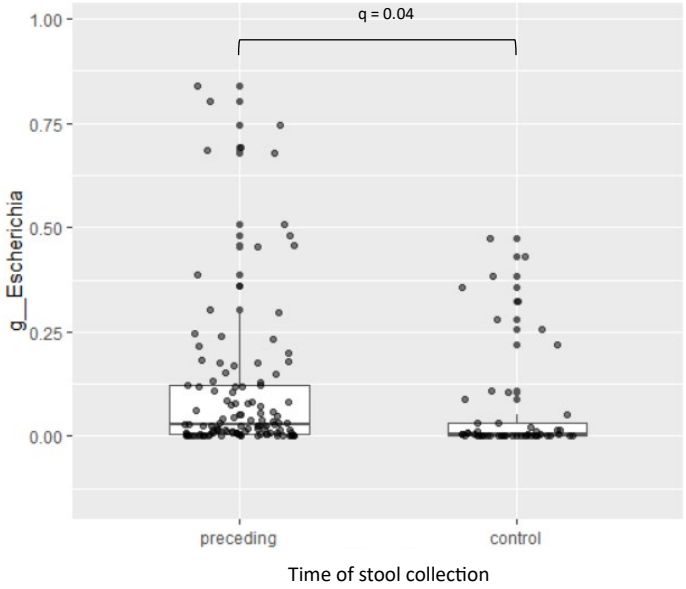


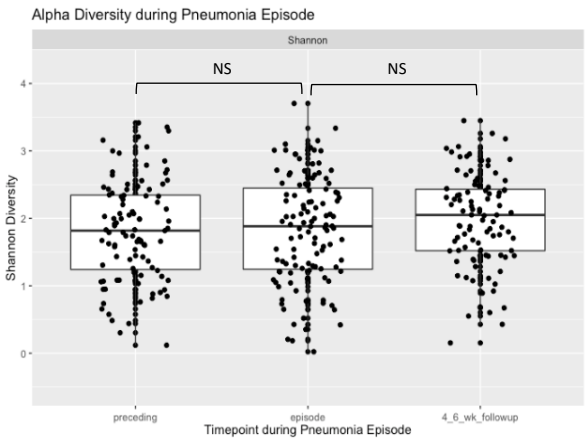
Figure 16A showed at a phylum level that Proteobacteria is significantly enriched at pneumonia episode timepoint compared to follow-up timepoint and controls after adjusting for age, method of stool collection and antibiotic exposure. Figure 16B showed significant enrichment of *E.coli* in pneumonia episodes compared to follow-up samples after adjusting for the method of stool collection, age, and antibiotic exposure. Figure 16C showed in a mixed-effects model adjusted for the method of stool collection, age, antibiotic exposure, mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, nutritional status (stunting) and daycare exposure, *E. coli* was significantly enriched in samples preceding a pneumonia episode compared to age-matched non-pneumonia samples (q=0.04).

Supplementary material

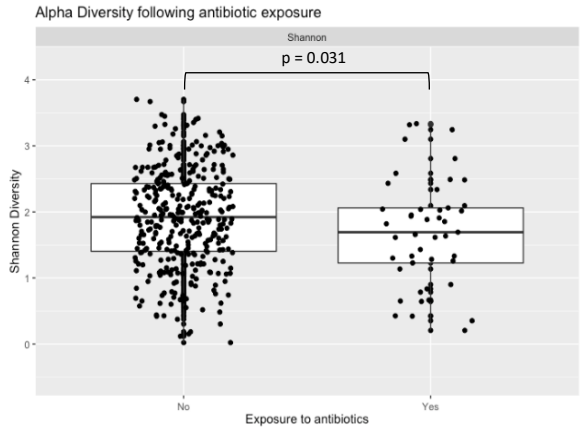
- Figure 17A, B: Shannon Alpha Diversity
- Figure 18: Beta diversity analysis of age groups
- Figure 19: Community composition at phylum level in each age group
- Figure 20A, B, C: Differentially abundant taxa between pneumonia timepoints

Figure 17: Shannon Alpha Diversity adjusted for method of stool collection (passed vs aspirated), mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, daycare exposure and exposure

A

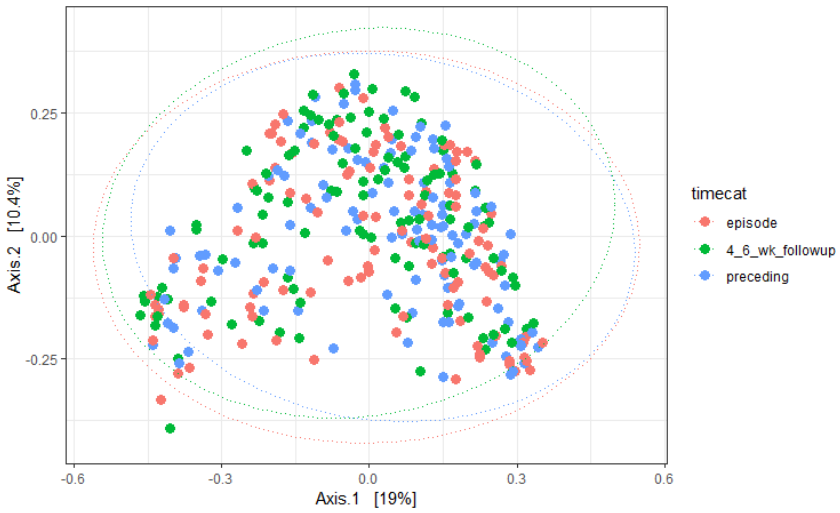


B



A: No difference in alpha diversity between the preceding, episode, and follow-up samples in the pneumonia group. B: Alpha diversity was significantly lower in samples exposed to antibiotics at the time of stool collection after adjusted for stool collection method and age.

Figure 18: Beta diversity analysis of age groups, adjusted for the method of stool collection, mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, daycare exposure and antibiotic exposure



Beta diversity analysis of pneumonia timepoints showed distinct clustering of preceding, episode, and follow-up samples.

Figure 19: Community composition at phylum level in each age group

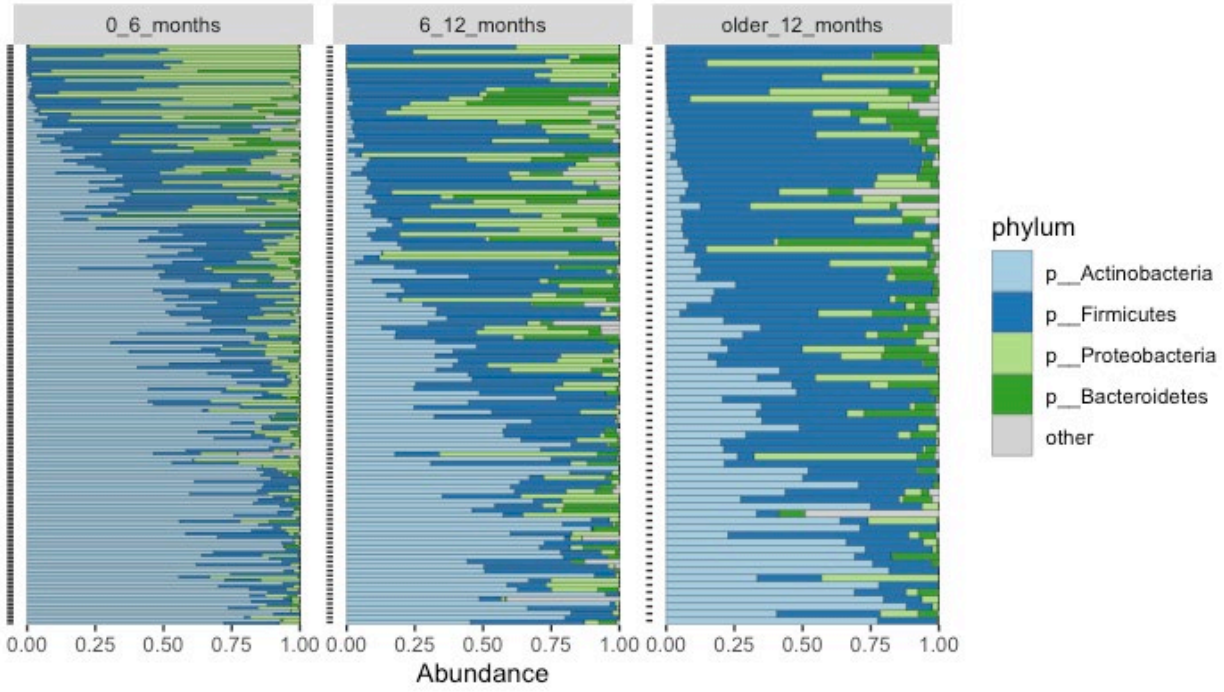
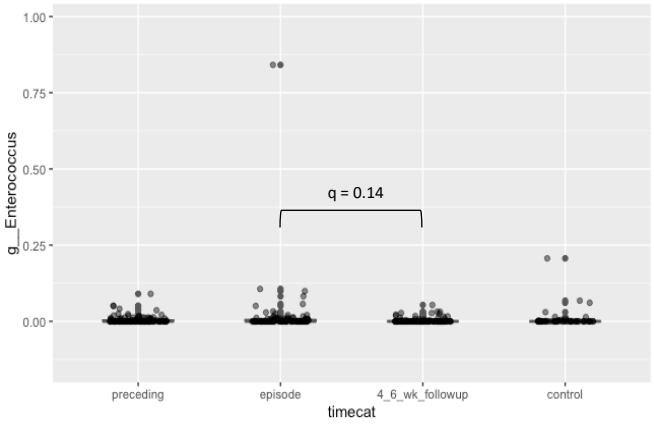


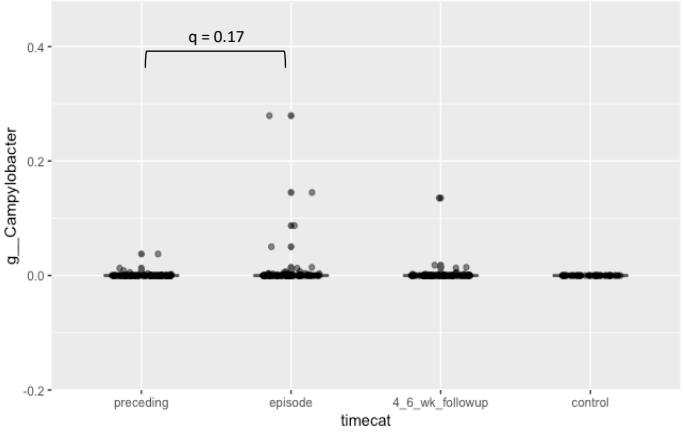
Figure 19 showed taxonomic relative abundance at phylum level in each age category.

Figure 20: Differentially abundant taxa between pneumonia timepoints (adjusted for age, method of stool collection and antibiotic exposure)

20A



20B



20C

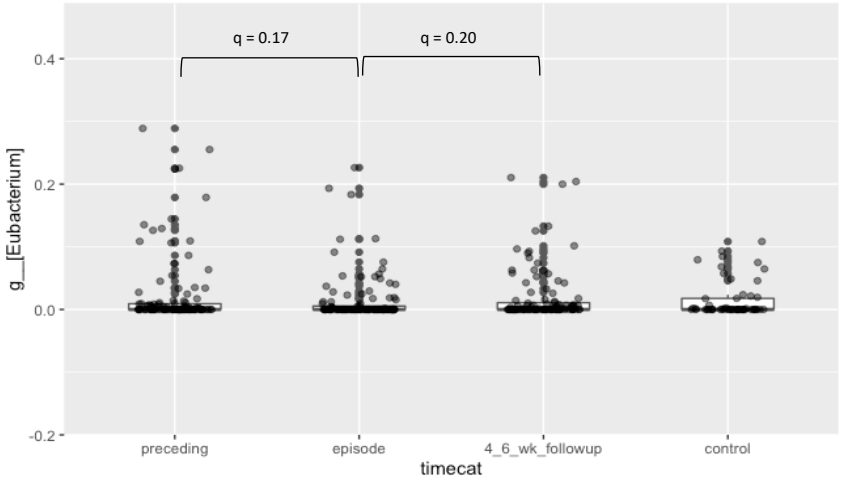


Figure 20: All models adjusted for the method of stool collection, age, and antibiotic exposure. A showed significant enrichment in Enterococcus during pneumonia episode compared to follow-up samples. B showed that Campylobacter was significantly enriched in episode compared to preceding samples. C showed that Eubacterium was significantly depleted during episode compared to preceding and follow-up samples.

Chapter 6: Summary and recommendations

The global prevalence of childhood malnutrition remains high, with LMICs being disproportionately affected. Despite some improvement in the prevalence of undernutrition globally, reduction in Africa has been slow. Children under 5 with malnutrition face a significantly higher risk of mortality during childhood. Specifically, undernutrition is an important risk factor for the development of childhood pneumonia and is associated with poor outcomes. Therefore, understanding the prevalence of malnutrition and factors associated with it is key to optimising child health. Concurrent with persistently high rates of undernutrition are rapidly rising rates of overnutrition and obesity on a global scale with many LMICs facing the double burden of malnutrition. Current interventions aimed at reducing the prevalence of pneumonia and malnutrition may have reached a therapeutic ceiling, creating a need for novel strategies to address disease burden.

There is growing evidence suggesting that early life exposures, including in-utero exposures and maternal health, have lasting effects on growth, the risk of non-communicable disease and nutritional outcomes throughout childhood and into adult life. Of particular concern is the rapidly expanding epidemic of obesity and metabolic disease. Investigating the impact of early life exposures on health throughout childhood may elucidate modifiable risk factors for the development of adverse nutritional, infectious, and metabolic outcomes in later life. The long-term effects of early life exposure may be mediated by epigenetic programming, and the gut microbiome. Robust collection of exposure data and biomarkers in birth cohorts and correlation of these with long term health outcomes will help elucidate potential targets for future therapeutic interventions.

A healthy microbiome during infancy and childhood is crucial for the normal development of the immune system and the prevention of nutritional, infectious, and inflammatory disease throughout life. There are several well-studied factors that are known to disrupt the healthy microbiome during childhood including mode of delivery (caesarian section versus normal vaginal delivery), duration of breastfeeding, and antibiotic exposure. Despite the excessively high rate of morbidity and mortality in African children, there is a significant under-representation of African microbiome studies in the literature. Therefore, the characteristics of a ‘normal’ microbiome in children residing in sub-Saharan Africa remain unknown. Reference databases that are used to classify bacterial sequences are based on Western populations, which further contributes to the significant under-characterisation of the African gut microbiome. Population-based microbiome studies in adults and children – like the American Gut Project – are required in African populations to better understand the healthy African

microbiome and could lead to the discovery of novel microbiome interventions aimed at preventing disease.

This body of research addresses significant gaps in knowledge regarding growth, feeding practices and the microbiome in South African children. Taken together, there are several modifiable maternal and infant risk factors contributing to poor growth during infancy. Despite a high prevalence of poor feeding practices there was no correlation with growth outcomes at 1 year. The gut microbiome, a previously understudied factor that contributes to health during childhood, was shown to be disrupted in children with stunting and potentially a risk factor for the development of childhood pneumonia through the microbiome-gut-lung-axis. Infant growth, diet (nutrient intake) and the microbiome are closely intertwined, and all contribute to health during childhood. This research helps identify modifiable factors that could be targeted to improve health outcomes in children.

Summary of results

This work provided novel data on growth, the prevalence of malnutrition in South Africa, and determinants of growth and malnutrition from birth through early childhood, by leveraging a unique very well-phenotyped birth cohort, the DCHS. In addition, the association of the gut microbiome with malnutrition and pneumonia was investigated providing novel data on the association of the gut microbiome with stunting and the development of childhood pneumonia.

Key findings are summarised below.

1. Prevalence of malnutrition and determinants (Chapter 2)

Malnutrition – both under and overnutrition – was highly prevalent in this LMIC population. Birthweight and infant growth were impacted by several modifiable maternal and infant factors. There was a high prevalence of low birth weight (15%, 115/792) and SGA infants (26%, 203/792) and a mean WFAZ score at birth below the WHO reference population (-0.5, IQR -1.3 to 0.1). Antenatal maternal smoking, alcohol consumption, and maternal height were important predictors of infant birthweight, and are also major determinants of early growth and intestinal microbiome composition.

Between two and six months, infants exhibited higher weight gain than height gain. The period of highest weight gain during infancy was between birth and six months. Median HFAZ remained negative at all time points, indicating that the infants had slower linear growth and remained shorter than infants from the reference WHO population. Stunting was highly prevalent in this study, ranging from 17% (115/681) of infants at two months to 13% (43/342) by 12 months. Birthweight, SES, and maternal antenatal alcohol and tobacco consumption were important determinants of the infant's longitudinal growth during the first year of life. The high prevalence of stunting was surprising given the study setting, with access to healthcare facilities, free maternal antenatal care, nutritional programs and a strong primary healthcare program. Several additional factors such as quality of diet and the gut microbiome may play crucial roles in the pathogenesis of stunting in this population.

Importantly, an emerging group of overweight and obese infants were observed, a global trend that disproportionately affects LMICs and has been associated with changing feeding patterns and lifelong adverse effects on health. At 12 months of age, 2% of the children were obese and 6% were overweight. Analysis of nutritional status in this birth cohort identified important determinants of birthweight and growth during infancy, in addition demonstrating the presence of overweight and obesity during early life.

2. Feeding practices from birth and association with growth outcomes (Chapter 3)

The study found lower rates of EBF for 6 months than previously reported in other South African studies and other LMICs, frequent early introduction of complementary foods, low dietary diversity, and low consumption of iron rich foods between 6 and 9 months. The WHO recommends EBF for 6 months and local recommendations among HIV-positive mothers evolved from exclusive formula to exclusive breastfeeding prior to the study. However, less than half of HIV-exposed infants were started on breast milk at birth, suggesting slow implementation. Many additional obstacles to EBF in South Africa remain including maternal perception of safety of breastfeeding in HIV-infected mothers, insufficient breast milk, concern about poor growth, marketing of breast-milk substitutes, maternal work, and lack of supportive work structures to promote breastfeeding.

There was a concerning pattern of high consumption of inappropriate foods, beginning as early as 6 months. The early introduction of complementary foods, and frequent, early consumption of inappropriate complementary foods raises concerns about the impact of such dietary practices on obesity and metabolic disease in later childhood and adult life. While the consumption of inappropriate foods (crisps and high sugar or high fat foods) during infancy was not significantly

associated with overweight or obesity at 12 months in this study, assessment of anthropometry at later timepoints will determine the impact of early-life consumption of inappropriate foods on overweight and obesity in later childhood.

The WHO Infant and Young Child Feeding (IYCF) indicators including minimum dietary diversity and consumption of iron-rich foods were not associated with growth outcomes at 12 months, suggesting that more sensitive indicators of dietary quality that better correlate with growth outcomes are required. Longer duration of breastfeeding was associated with a lower HFAZ at 1 year. This may be due to transient slowing in linear growth at 12 months. Recent studies suggest that the quality of breastmilk (i.e., type of HMOs present in breast milk) and the ability of the infant microbiome to metabolise HMOs in breastmilk, may be associated with growth outcomes in infants.

3. The early life gut microbiome and its association with growth (Chapter 4)

A surprisingly high rate of stunting was observed in this birth cohort despite access to public health programs supporting nutrition. Similarly, several global initiatives aimed at treating stunting, including maternal/pregnancy interventions, dietary modifications and micronutrient supplementation have resulted in minimal or no improvement in growth outcomes, suggesting that nutritional supplementation alone is not sufficient to address the underlying pathogenesis of stunting (1,2). A growing body of evidence shows that the gut microbiome contributes to the development and persistence of malnutrition (3–5). A distinct microbiome pattern was associated with stunted children compared with those with normal linear growth in this birth cohort. Severe stunting was associated with a significantly lower alpha diversity compared with the normal height group and higher HAZ was positively associated with higher alpha diversity. Overall bacterial community composition in samples from children with severe stunting was significantly different to those of normal height on beta diversity analysis. Taxonomic composition was mostly similar between the groups at phylum level. However, at genus level, *Ruminococcus,* and at species level, *Ruminococcus gnavus,* were significantly depleted in the stunted group compared with the normal height group. The findings from this study support the growing evidence that important butyrate-producing organisms within the class *Clostridia* are depleted in children with stunting. In addition, studies have begun unravelling the underlying causal mechanisms of specific microbes or consortia of microbes, including R. *gnavus*, that could lead to novel approaches to treat stunting with defined microbiome therapies in addition to feeding.

4. The association of the gut microbiome with childhood pneumonia (Chapter 5)

Intestinal microbiome dysbiosis was present during childhood pneumonia episodes characterised by expansion of *E. coli*, and depletion of SCFA-producing microbes. Importantly, dysbiosis was present prior to the onset of pneumonia compared with non-pneumonia controls, with expansion in *E. coli* in samples preceding the onset of symptoms, supporting the gut–lung axis concept that gut microbiome dysbiosis is a risk factor for pneumonia. In this robust mixed-effects model adjusted for the method of stool collection, age, antibiotic exposure, mode of delivery, exclusive breastfeeding, sex, prematurity, HIV exposure, nutritional status (stunting), and daycare exposure, *E. coli* was significantly enriched in samples preceding a pneumonia episode compared with age-matched non-pneumonia samples (q=0.04). In a similarly adjusted model, *E. coli* was significantly enriched in pneumonia episode samples compared with age-matched non-pneumonia samples (q=0.005). These findings suggest that compared with healthy, age-matched, non-pneumonia controls, microbiome dysbiosis was present prior to onset of pneumonia symptoms, characterised by asymptomatic bloom in *E. coli*. These findings support the 'gut–lung' axis thesis, that gut microbiome dysbiosis is an important risk factor in the pathogenesis of RTI during childhood and provides a potential target for microbiome therapeutics to prevent childhood pneumonia.

Research in context

Impact

This body of research addressed significant gaps in the literature including risk factors associated with longitudinal growth and dietary practices among South African children. Furthermore, no studies have investigated the association of the gut microbiome with growth longitudinally in South African children. To date, no studies have investigated the gut microbiome longitudinally, including prior to pneumonia onset, and none from LMIC settings where disease burden is highest. The findings observed in the novel studies presented in this thesis may inform future interventional studies aimed at addressing the burden of disease in children residing in low resourced settings.

Strengths and limitations

This study had several strengths. First, the prospective design with high cohort retention and longitudinal measurements of multiple parameters of infant growth by trained study staff, together with the collection of comprehensive exposure data including maternal antenatal data, provided a

rich, high-quality dataset for statistical modelling. Second, the comprehensive longitudinal collection of feeding information in the form of food frequency questionnaires provided unique insight into feeding patterns among children in the cohort. Third, the longitudinal characterisation of the intestinal microbiome in children with stunting and childhood pneumonia, and the comparison with healthy controls. The cohort design with frequent longitudinal sampling enabled the characterisation of the microbiome before the onset of symptomatic disease, which is crucial in differentiating cause versus effect in microbiome research.

Limitations of this study included the duration of follow-up in this analysis. Growth, nutritional status, and feeding practices were evaluated through infancy. Longer follow-up would be helpful to determine the impact of early life factors on growth and nutritional outcomes in later childhood. The birth cohort is ongoing with the opportunity to perform further analysis in the future. Another limitation was the method of dietary information collection. While food frequency questionnaires are a useful and less burdensome tool for collecting dietary information, they do not provide sufficiently detailed dietary information to enable the calculation of micro- and macronutrient content. In this study, the food frequency questionnaire did not provide sufficient information to calculate some of the WHO IYCF indicators. Further analysis of the correlation of WHO IYCF indications with additional timepoints i.e., before and after 1 year of age will need to be performed in future studies. Lastly, there were several limitations regarding the microbiome analysis. Due to the challenges associated with the collection of stool samples in children, two different methods of stool collection were used (passed stool versus rectal aspirates). To minimise the effect of the method of sample collection, all statistical analyses adjusted for method of sample collection. For analysis of the microbiome in stunting, approximately 30% of participants only had one stool sample included in the analysis. Instead of removing these samples from the analysis, they were included to increase the quality of the microbiome analysis models. Ideally, all stool samples in the study should be sequenced to characterize the microbiome of this population in detail and improve confidence in the longitudinal data analysis. However, this is not feasible due to the costs associated with performing microbiome sequencing at this time but may be possible in the future as sequencing costs and funding improve.

Recommendations

Future research

1. Interventional studies that focus on improving the modifiable risk factors associated with poor nutritional outcomes, including maternal smoking, alcohol consumption, maternal nutrition, and birth outcomes, will help confirm the causal nature of these risk factors.

2. The establishment of a South African and, thereafter, a broader African universal donor stool bank, would help catalyse descriptive and interventional microbiome research on the African continent.

3. There was a distinct microbial pattern in children with stunting compared with those with normal linear growth. Specifically, there was significant depletion of *R. gnavus*, a bacterial species, which is a known SCFA producer and has been shown to improve growth in animal models. Further work in animal models to identify and characterise the *R. gnavus* strain that was found to be depleted in stunted children, using culture and metagenomic sequencing, may enable the development of future interventional studies in children with malnutrition. The strain could be administered to humanised mice models of malnutrition to determine safety and efficacy prior to administration in humans.

4. Given the novel findings of dysbiosis prior to onset of pneumonia using 16S rRNA gene amplicon sequencing, metagenomic sequencing in pneumonia cases and non-pneumonia controls should be done to facilitate the identification of strain-level differences and differences in functional metabolic pathways between the two groups. Additionally, immune profiling, serum, and stool metabolomics may help elucidate the mechanisms underlying the gut–lung axis.

Policy implications

1. Supporting maternal nutrition including the provision of food supplementation and multiple micronutrients from the pre-conception period, throughout pregnancy, and during the post-partum period is crucial to improve foetal growth, reduce LBW and small for gestational age pregnancy outcomes, and improve infant and childhood nutritional outcomes.

2. Public health programs are needed to educate mothers about the detrimental effects of tobacco and alcohol use on birth outcomes and subsequent infant and child growth. These should include including effective cessation programs that are made accessible to pregnant woman and women of child-bearing age.

3. Education and support are needed to promote EBF for 5–6 months and continued breastfeeding beyond 6 months (in addition to complementary feeding) in infants and children younger than 5 years. While exclusive breastfeeding may not reduce the prevalence of stunting and wasting, it does reduce infections, specifically diarrheal infections, which contribute to malnutrition and mortality in children residing in LMICs. National policies to promote breastfeeding – including maternity leave and providing workplace resources to support breastfeeding together with restriction for use of formula feeds – should be introduced.

4. National policies to limit marketing of unhealthy foods and the introduction of sugar-sweetened beverage taxes to reduce the growing epidemic of overweight and obesity should be considered.

5. Healthcare institutions should consider implementing policies that minimise disruption to the intestinal microbiome during childhood. These may include promoting normal vaginal deliveries and reducing the number of elective caesarean sections and promoting exclusive breastfeeding. Importantly, implementation of antibiotic stewardship programs will be crucial for the reduction of unnecessary antibiotic exposure during childhood.

Conclusion

Malnutrition – encompassing both undernutrition, overweight, and obesity – remains a significant global health concern contributing to morbidity and mortality throughout life. Interventions during the antenatal (in utero), infancy, and early childhood periods are crucial to preventing adverse outcomes associated with childhood malnutrition. Novel strategies that complement food supplementation are required to address the burden of malnutrition. The findings from this research conducted in a large birth cohort are relevant to other LMIC settings globally. The thesis presented novel epidemiological data on nutritional status during infancy; highlighted the impact of maternal and in-utero factors adversely affecting growth during infancy; and demonstrated previously underestimated poor feeding practices during infancy. This work also included the first published

analysis of the intestinal microbiome in children with stunting and childhood pneumonia, identifying novel findings that could inform future preventative or therapeutic interventions globally.

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1. Prendergast AJ, Humphrey JH. The stunting syndrome in developing countries. Paediatr Int Child Health. 2014;34(4):250–65.

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5. Blanton LV, Charbonneau MR, Salih T, Barratt MJ, Venkatesh S, Ilkaveya O, et al. Gut bacteria that rescue growth impairments transmitted by immature microbiota from undernourished children. Science. 2015;33(4):395–401.

Appendix

Ethical approval, UCT Human Research Ethics Committee

HUMAN RESEARCH ETHICS COMMITTEE
18 SEP 2023
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN

UNIVERSITY OF CAPE TOWN
FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report / Renewal

HREC office use only (FWA00001637; IRB00001938) This serves as notification of annual approval, including any documentation described below.			
☐ Approved	Annual progress report	Approved until/next renewal date	30.9.2024
☐ Not approved	See attached comments		
Signature Chairperson of the HREC/ Designee		Date Signed	6/9/2023

Note: Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za.
Please clarify your plan for research-related activities during COVID-19 lockdown.
Please use the latest form found on our website:
http://www.health.uct.ac.za/fhs/research/humanethics/forms

Comments to PI from the HREC
Thank you for the deviation document

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	20 August 2022		
HREC REF Number	739/2016	Current Ethics Approval was granted until	28 Feb 2021
Protocol title	Nutrition, micronutrient deficiency, the intestinal microbiome and childhood pneumonia in South African Infants		
Protocol number (if applicable)			
Are there any sub-studies linked to this study?	☐ Yes	☑ No	
If yes, could you please provide the HREC Reference number for all sub-studies? **Note:** A separate FHS016 must be submitted for each sub-study.			
Principal Investigator	Prof Heather Zar		

(Note: Please complete the Closure form (FHS010) if the study is completed within the approval period)

Authorization letters from co-authors

School of Public Health
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Date: 10 January 2024

Professor and Chairman: Faculty Doctoral and Masters Committee
Faculty of Health Sciences
University of Cape Town

Dear Prof Morrow,

Permission letter as co-author with Dr. Shrish Budree to include publications in PhD submission.

I was a co-author with Dr. Shrish Budree on the following publications:

1. Budree S, Stein DJ, Brittain K, Goddard E, Koen N, Barnett W, Myer L, Zar HJ. Maternal and infant factors had a significant impact on birthweight and longitudinal growth in a South African birth cohort. Acta Paediatr. 2017 Nov;106(11):1793-1801. doi: 10.1111/apa.14015. Epub 2017 Sep 4.
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I concur that Dr. Budree (student number BDRSHR001) was the lead researcher and primary author of the manuscript, and I give permission for these publications to be included in his PhD thesis.

Kind regards,

Landon Myer
Head: Division of Epidemiology and Biostatistics

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13 December 2023
Professor and Chairman: Faculty Doctoral and Masters Committee
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Dear Prof Morrow,

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I concur that Dr. Budree (student number BDRSHR001) was the primary author of the manuscript, and I give permission for these publications to be included in his PhD thesis.

Kind regards,

Kirsty Brittain
Centre for Child and Adolescent Lung Health Unit
University of Cape Town Lung Institute

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Date: 12th December 2023

Professor and Chairman: Faculty Doctoral and Masters Committee
Faculty of Health Sciences
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Dear Prof Morrow,

Permission letter as co-author with Dr. Shrish Budree to include publications in PhD submission

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Kind regards,

Dr Elizabeth Goddard
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University of Cape Town

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Kind regards,

Mark Nicol
Professor in Microbiology, University of Western Australia

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Date:

Professor and Chairman: Faculty Doctoral and Masters Committee
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University of Cape Town

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Kind regards,

Majdi Osman
Chief Medical Officer, OpenBiome

UNIVERSITY OF CAPE TOWN
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12 December 2023

Professor and Chairman: Faculty Doctoral and Masters Committee
Faculty of Health Sciences
University of Cape Town

Dear Prof Morrow

Re: Permission letter as co-author with Dr. Shrish Budree to include publications in PhD submission

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I concur that Dr Budree (student number: BDRSHR001) was the lead researcher and primary author of this manuscript; and I give permission for this publication to be included in his PhD thesis.

Please do not hesitate to contact me should any further information be needed in this regard.

Sincerely,

A/Prof Nastassja Koen (MBChB, PhD)
Associate Professor and Head: Division of Psychopharmacology and Biological Psychiatry
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18 December 2023

Professor and Chairman: Faculty Doctoral and Masters Committee
Faculty of Health Sciences
University of Cape Town

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Kind regards,

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Department of Pathology, University of Cape Town

LETTER 1 (1)

11.12.2023

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Date: 11 Dec 2023

Professor and Chairman: Faculty Doctoral and Masters Committee
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University of Cape Town

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Kind regards,

Tommi Vatanen
Associate Professor
Computational Microbial Ecology group
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December 12, 2023

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Yours sincerely

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Kind regards,

Chief Dietitian
Head of Dietetics Department
Red Cross War Memorial Childrens Hospital